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NGFI-B redox sensitivity and regulation of mitochondrial bioenergetics

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NGFI-B redox sensitivity and regulation of mitochondrial bioenergetics

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Dedication

To my family and my husband Sean for all of their love and support

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NGFI-B redox sensitivity and regulation of mitochondrial bioenergetics

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Changes in intracellular redox homeostasis are implicated in both normal cell signaling and as pathophysiological mechanisms contributing to a variety of age-related diseases, including diabetes, atherosclerosis, neurodegenerative conditions, and cancer. Though a variety of well described mechanisms exist to counterbalance the overproduction of cellular oxidants and maintain optimal intracellular redox poise, the understanding of the mechanism(s) through which cellular redox homeostasis regulates cell signaling functions is less well understood. Here, we demonstrate that signaling by the immediate early gene / orphan nuclear hormone receptor NGFI-B (Nur77, TR3), which functions pleiotropically in the regulation of cell growth, metabolism, differentiation and death in diverse tissues, is redox-regulated at both the level of induction and NGFI-B-dependent gene transcription. Using co-immunoprecipitation experiments in cells, we also identified a novel interaction between NGFI-B and the cytoplasmic thiol-reducing catalyst thioredoxin1 (Trx1), that, similar to DTT, blocks NGFI-B-dependent gene expression in a manner that depends on the Trx1 active site cysteines. Together these observations add NGFI-B-dependent gene expression to a growing portfolio of transcription factor pathways that are redox-regulated.

NGFI-B, in addition, appears to regulate the mitochondrial membrane potential in L6 skeletal myoblasts. NGFI-B is indispensible for T-cell receptor-mediated apoptosis and induces cell death in a variety of cell types in response to diverse pro-apoptotic stimuli. Like p53, translocation of NGFI-B from the nucleus to the mitochondria may be a critical aspect of its pro-apoptotic function. Interestingly, we found that enforced NGFI-B expression in L6 skeletal muscle myoblasts led to a significant decrease of MMP that peaked 48hr after transfection and did not require a cell death-inducing stimulus. Moreover, NGFI-B transfected cells had no increase in mitochondrial cytochrome C release despite loss of MMP at 48 hr. Combined, these data suggest that loss of MMP in muscle cells may be an early event in the apoptotic process regulated by NGFI-B. This, along with the redox regulation of NGFI-B, provides unique evidence of a relationship between the mitochondria, mitochondrial by-products, ROS, and the regulation of and by the transcription factor NGFI-B.

List of Abbreviations

8-OH-dG	8-hydroxy-2'deoxyguanosine
ADP	Adenosine Diphosphate
AF-1	Transactivation domain
AHPN	6-(3-(1-adamantyl)-4hydroxyphenyl)-2-naphthalene carboxylic acid
AIF	Apoptosis Inducing Factor
AMP	Adenosine Monophosphate
AMS	4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid
ANT	Adenine Nucleotide Translocase
AP-1	Activator Protein 1
ApoE	Apolipoprotein E
ATP	Adenosine Triphosphate
β-AR	Beta-Adrenergic Receptor
Bcl-2	B Cell Lymphoma-2
Ca	Calcium
cAMP	Cyclic Adenosine Monophosphate
CREB	cAMP Response Element Binding
CspA	Cyclosporin A
Cu/Zn SOD	Copper/Zinc Superoxide Dismutase
Cys (C)	Cysteine
DBD	DNA Binding Domain
DMEM	Dulbecco Modified Eagle's Minimal essential medium
DMSO	Dimethyl Sulfoxide
DNP	Dinitrophenol
DTT	Dithiothreitol
EGF	Epidermal Growth Factor
eNOS	Epithelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ER	Estrogen Receptor
ERK	Extracellular signal-Regulated Kinase
ETC	Electron Transport Chain
EV	Empty Vector
FADH2	Flavin Adenine Dinucleotide (reduced form)
Fe	Iron
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GC	Glucocorticoid Receptor
GFP	Green Fluorescent Protein
GSH	Glutathione
GTPase	Guanosine Triphosphatase
H_2O_2	Hydrogen peroxide
HBSS	Hanks Buffered Salt Solution
Hek293T	Human embryonic kidney 293T cells

Hif1α	Hypoxia Inducible Factor 1 alpha
HUVEC	Human Umbilical Vein Endothelial Cells
IB	Immunoblot
IEG	Immediate Early Gene
IgG	Immunoglobulin G
IM	Intermembrane
iNOS	Inducible Nitric Oxide Synthase
IP	Immunoprecipitation
JNK	c-Jun N-terminal Kinase
KCl	Potassium Chloride
LBD	Ligand-Binding Domain
LDL	Low Density Lipid
LPS	Lipopolysaccharides
LXR	Liver X Receptor
MAPK	Mitogen Activated Protein Kinase
MEKK-1	MAP Kinase Kinase 1
Mn SOD	Manganese Superoxide Dismutase
MMP	Mitochondrial Membrane Potential
MPTP	Mitochondrial Permeability Transition Pore
mtDNA	Mitochondrial DNA
Nac	N-acetyl cysteine
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBRE	NGFI-B Response Element
NES	Nuclear Export Signal
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
NGFI-B	Nerve Growth Factor Inducible protein B
NHR	Nuclear Hormone Receptor
NLS	Nuclear Localization Signal
Nor-1	Neuron-derived Orphan Receptor 1
NOS	Nitric Oxide Synthase
NOX	NADPH Oxidase
NR4A	Nuclear Receptor Subfamily 4, group A
NuRE	Nur77 Response Element
Nurr1	Nuclear Receptor Related 1 protein
OM	Outer Membrane
PAI-1	Plasminogen Activator Inhibitor-1
PC12	Pheochromocytoma 12 cells
PDGF	Platelet Derived Growth Factor
pKa	Acid constant
P/M	Pyruvate/Malate
PMA	Phorbol 12-myristate 13-acetate

PTEN	Phosphatase and Tensin homolog
PTP	Protein Tyrosine Phosphatase
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
Ser (S)	Serine
SOD	Superoxide Dismutase
SO2H	Sulfinic acid
SO3H	Sulfonic acid
TCA	Trichloroacetic acid
TCR	T-cell Receptor
TGFβ	Transforming Growth Factor Beta
TMRM	Tetramethylrhodamine, methyl ester, perchlorate
TNF-α	Tumor Necrosis Factor- alpha
TOR	Target of Rapamycin
Trx1	Thioredoxin 1
UCP	Uncoupling Protein
UPR	Unfolded Protein Response
UV	Ultra Violet
VDAC	Voltage-dependent Anion Channel
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cells
XOR	Xanthine Oxidoreductase

Table of Contents

List of Tables

List of Figures

List of Illustrations

Chapter 1: Introduction

1.1 BACKGROUND

Cellular reactive oxygen species including superoxide, peroxides, and hydroxyl radicals, have risen over the last several years from their infamous status as capricious toxic byproducts of aerobiosis to requisite mediators of a variety of cell fate specification signaling pathways (Cross, Halliwell et al. 1987). Decades old work dealing with the mechanisms linking ROS generation to neurodegenerative, inflammatory, and neoplastic diseases has informed the more recent view that cells also intentionally produce, sense, and destroy oxidants as part of a novel second messenger signaling system in cells (Allen and Tresini 2000; Balaban, Nemoto et al. 2005; D'Autreaux and Toledano 2007; Genestra 2007). Although the biochemical mechanisms of oxidative damage to lipids, proteins, and DNA in the context of high concentrations of oxidative species (e.g. "oxidative stress") are well described, comparably less is known about the mechanisms and targets of physiological oxidative signaling.

Pioneering work beginning in the mid 1990's established that ROS are important cell growth messengers. It was initially discovered that tyrosine kinase receptor-activated ROS production promotes growth factor-induced cell growth through the inactivation of growth inhibitory tyrosine phosphatases (Lee, Kwon et al. 1998; Rhee, Bae et al. 2000; Kwon, Lee et al. 2004). Other reports demonstrated that ROS also control cell functions by directly modifying the activities of transcription factors; a notable example is the induction of antioxidant genes in response to activation of the transcription factor complex AP-1 (Allen and Tresini 2000). In yeast, the orthologous Yap1 transcription factor was shown to rapidly redistribute to the nucleus and regulate gene expression in

response to the oxidation of protein disulfide bonds (Wood, Storz et al. 2004). Since these initial observations, a small but growing list of redox sensitive transcription factor signaling networks has been identified.

With this new evidence revealing that ROS are crucial signaling molecules more attention is being placed on the mitochondrial production of ROS, the major source of endogenous ROS within the cell. A variety of factors exist regulating the production of mitochondrial ROS, including uncoupling proteins, calcium signaling and the MPTP, as well as nutrient sensing transduction pathways (Garlid, Jaburek et al. 2001; Halestrap, McStay et al. 2002; Sabatini 2006). It is now clear that mitochondrial bioenergetics is far more involved in the regulation of cellular processes than simply maintaining energy production and crucial to further our understanding of the mechanisms by which cellular metabolism regulates cell fate decisions.

The mitochondrial membrane potential (MMP) was once thought to solely provide the electrochemical gradient necessary for the production of ATP (Mitchell 1979) however the MMP is now considered important for both signal transduction and apoptosis (Huttemann, Lee et al. 2008). Loss of the MMP occurs when an increase in proton conductance thereby dissipates the difference between protons within the intermembrane space and those found in the mitochondrial matrix. This depolarization of the MMP has been demonstrated to increase the transfer of electrons from the donors and acceptors of the ETC, thereby reducing the likelihood that electrons slip and create superoxide (Korshunov, Skulachev et al. 1997; Liu 1999). Loss of MMP and the means that regulate this loss could therefore be directly involved in altering redox poise within the cell. It is necessary that future redox signaling research not only continue to identify the downstream targets of ROS signaling, but also identify the variety of mechanisms in place regulating the production of the signaling molecule itself.

1.2 REACTIVE OXYGEN SPECIES (ROS)

The early incorporation of the energy producing bacteria or mitochondria into a single cell brought with it both benefits as well as draw backs (Balaban, Nemoto et al. 2005). Although oxygen consumption and mitochondrial respiration are vital to an organism's survival, the metabolic by-products have been shown to cause damage to the cell lending to the aging of an organism and several degenerative diseases. Reactive oxygen species have long been regarded as destructive by-products of aerobic respiration capable of irreversibly damaging lipids, proteins, and DNA. While that may still be true when their levels go unchecked, evidence over the past decade has shown that ROS also act as a second messengers capable of regulating cell function (D'Autreaux and Toledano 2007). It has now become more and more clear that the antioxidant system once thought to be our only line of defense against these damaging by-products actually acts in concert with oxidants to regulate a variety of cellular processes from proliferation to cell death.

1.2.1 Types of ROS

Oxidants or ROS are molecules or ions generated by the incomplete one electron reduction of oxygen. Better known ROS include superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrate. ROS are highly reactive because they carry an unpaired valence shell electron. The reactivity of the various oxidants depends on their production within the cell, the presence of enzymes which regulate their conversion to less reactive species, and their rate of oxidation of other molecules within the cell, such as proteins, lipids, and DNA.

Super Oxide Anion	$O_2 + e^- \rightarrow \mathbf{O_2^{-\bullet}}$
Hydrogen Peroxide	$HO_2^{\bullet} + e^- + H \rightarrow H_2O_2$
Hydroxyl Radical	$H_2O_2 + e^- \rightarrow OH^- + {}^{\bullet}OH$
Fenton Reaction	$H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^{\bullet}OH + Fe^{3+}$
Nitric Oxide	L-Arginine + $O_2 \rightarrow {}^{\bullet}\mathbf{NO}$ + L-Citrulline
Peroxynitrite	$O_2^{-\bullet} + {}^{\bullet}NO \rightarrow \mathbf{ONOO^-}$

REACTIVITY: $O_2^{-\bullet} < H_2O_2 < {}^{\bullet}OH$

DIFFUSION: $H_2O_2 >>> O_2^{-\bullet} = {}^{\bullet}OH$

Table 1: Equations representing the production of endogenous reactive oxygen species.

Superoxide is the most prevalent form of ROS and is considered the "primary" ROS giving rise to other forms of "secondary" ROS. Superoxide is produced throughout the cell by metabolic respiration, NADPH oxidase isoforms, or by irradiation of molecular oxygen. While it is the most abundant oxidant within the cell it is tightly regulated and quickly dismutated to hydrogen peroxide due to the presence of cytosolic and mitochondrial superoxide dismutases. The rate (10^3 $M^{-1}s^{-1}$) at which superoxide could oxidize a thiol moiety is insignificant compared to the rate (> 10^9 $M^{-1}s^{-1}$) at which it is converted to hydrogen peroxide (Forman and Fridovich 1973). No direct targets of superoxide have been demonstrated *in vivo*, however, its connection to signaling has been described. It is highly likely that the signaling role of superoxide is attributed to its role as the precursor to hydrogen peroxide.

The hydroxyl radical is most notably generated from the Fenton reaction when hydrogen peroxide interacts with metal ions such as iron (Fe^{2+}). It is considered one of the most reactive of the oxidant species generated within the cell, with an extremely short half life of approximately 10^{-9} s (Pastor, Weinstein et al. 2000). It shows no specificity and reacts with almost any biological molecule in its path. While this makes it a poor second messenger, the hydroxyl radical has been implicated in some signaling pathways involving lipid peroxidation such as 4-hydroxy-2-nonenal and the release of isoprostanes (Mohler, Franklin et al. 1996; Levonen, Landar et al. 2004; Rinna and Forman 2008). However, these markers of ROS signaling by the hydroxyl radical are often associated with oxidative stress and damage and thus hydroxyl radicals are not widely considered physiologically relevant signaling mediators.

Hydrogen peroxide is considered the most likely physiological form of ROS, aside from nitric oxide, to serve as second messenger within a signaling cascade. As stated before hydrogen peroxide is most often generated from the dismutation of the superoxide ion. As a result, hydrogen peroxide is regulated and limited by the same spatial and temporal generation of superoxide as well as the antioxidant catalase which converts hydrogen peroxide to water and oxygen. Hydrogen peroxide is a non-polar molecule and can therefore cross membranes (Forman, Maiorino et al.). The main means of protein oxidation generated by hydrogen peroxide is on specific thiols or cysteines. These cysteines are often referred to as reactive cysteines because of their decreased pKa and residence near basic amino acids that support the thiolate anion form (Barford 2004). Since the discovery of hydrogen peroxide-dependent thiol oxidation other amino acids such has methionine have also been shown to be oxidized (for extensive review see (Vogt 1995; Hoshi and Heinemann 2001; Stadtman, Moskovitz et al. 2003).

Nitric oxide is the most established reactive oxygen species in cell signaling, winning "Molecule of the Year" in 1992 by *Science Magazine*. It is produced endogenously by the nitric oxide synthase enzymes using NADPH, L-arginine, and oxygen and plays a crucial signaling role in a variety of tissues within the cell (Palmer and Moncada 1989). Nitric oxide is highly reactive with a half life of only a few seconds and like hydrogen peroxide can diffuse freely across membranes making it an ideal paracrine and autocrine signaling agent (Moncada and Higgs 1991). The involvement of nitric oxide signaling has been well established in vascular endothelial dilation (Ignarro, Cirino et al. 1999). Similar to hydrogen peroxide nitric oxide directly modifies protein targets via S-nitrosylation on specific amino acids such as cysteines (Hess, Matsumoto et al. 2005). This post-translational modification of target proteins has been shown to regulate gene expression, energy production, and a variety of other cellular processes (Moncada, Palmer et al. 1991). When the production of superoxide and nitric oxide occur within close proximity to one another with in the cell the potent reactive oxygen species peroxynitrite is formed. Like the hydroxyl radical, peroxynitrite is often extremely damaging and associated with oxidative stress conditions for which more superoxide is present within the cell.

1.2.2 Generators of ROS

All organisms living under aerobic conditions have several enzyme systems that produce reactive oxygen species, including mitochondrial, peroxisomal, oxidoreductases, and a variety of cytoplasmic oxidases.

1.2.2.1 Mitochondria

The majority of oxidants are produced during aerobic respiration when electrons slip from the electron transport chain. This process is thought to mediate the production of ~80-90% of all basal oxidants. Molecular oxygen is the substrate for cytochrome oxidase (complex IV) and the terminal acceptor of electrons that flux across the mitochondrial respiratory chain during oxidative phosphorylation. However, electrons can also be released from ubisemiquinone binding sites in complexes I and III and non-enzymatically reduce oxygen to generate the reactive oxidant superoxide, which is rapidly converted to hydrogen peroxide by the mitochondrial and cytoplasmic isoforms of superoxide dismutase (Mn SOD and Cu/Zn SOD, respectively). Topologically, superoxide released from complex I is thought to occur mainly in the mitochondrial matrix, whereas complex III-generates superoxide in the mitochondrial intermembrane space (St-Pierre, Buckingham et al. 2002). Evidence also suggests respiratory complex II, glycerol-1-phosphate dehydrogenase, and dihydroorotate dehydrogenase may also contribute to the production of mitochondrial ROS (Krungkrai 1991; McLennan and Degli Esposti 2000; Lenaz 2001). While the production of ROS by the mitochondria and the part these metabolic by-products play in damaging the cell has been established, the exact role mitochondria ROS plays in signal transduction remains unclear.

1.2.2.2 Other generators of endogenous ROS

Other sources of endogenous ROS production include the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzymes, the xanthine oxidoreductase (XOR), which is involved in purine catabolism, and the organelle known as the peroxisome. These oxidant producing enzymes and organelles are located in the majority of cell types within an organism but serve a wide variety of cell-type specific functions.

The NADPH-dependent enzymes include the family of NADPH oxidases (NOX1-4), as well as the members of the nitric oxide synthase (nNOS, eNOS, and iNOS) family. The NOX family of enzymes was originally discovered for their role in phagocytic cells and the creation of superoxide for the defense against foreign organisms. It was not until their discovery in non-phagocytic tissues such as bovine arterial smooth muscle, myocytes, and endothelium (Mohazzab, Kaminski et al. 1994) that researchers began to describe a role for these enzymes in redox-signaling cascades. While superoxide remains a fairly poor second messenger, the time and spatial release associated with the NOX enzymes makes them excellent generators of ROS for cell signaling. NOX-generated superoxide has been implicated in the ROS bursts associated with growth factors such as platelet derived growth factor (PDGF) and transforming growth factor β (TGFβ) induced protein tyrosine phosphorylation cascades (Abid, Kachra et al. 2000; Hu, Ramachandrarao et al. 2005). The mechanism by which the NOX family produces superoxide involves a small Rho guanosine triphosphatase (GTPase), usually Rac1 or Rac2, assembling with five phagocytic oxidase subunits which utilize electrons from NADPH to produce the free radical superoxide: $NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^{\circ-} + H^+$ (reviewed extensively in Morel, Doussiere et al. 1991; Groemping and Rittinger 2005; Ushio-Fukai 2006; Bedard and Krause 2007). Superoxide is then dismutated further by a superoxide dismutase to the more oxidizing redox second messenger hydrogen peroxide (H_2O_2). The time and spatial regulation of the NOX-dependent production of superoxide depends on both the assembly of the subunits (NOX1,2) or the transcription of the oxidase itself (as for NOX4) as well as their location within the cell (Thannickal and Fanburg 2000). NOX enzymes have now been described at the plasma membrane of cells as well as within the nucleus and at the endoplasmic reticulum (Ushio-Fukai 2006).

Nitric Oxide synthase, which is involved in a wide variety of cell signaling pathways, converts L-arginine and NADPH to nitric oxide and L-citrulline. There are three known NOS family members: neuronal nNOS (NOS-1), cytokine inducible iNOS (NOS-2) and endothelial eNOS (NOS-3). The eNOS is anchored to the plasma membrane of endothelial cells and has primarily been characterized for its role in the production of nitric oxide for vasodilation of blood vessels. Mutations and deletions of eNOS result in disorders such as atherosclerosis and erectile dysfunction (Hurt, Musicki et al. 2002; Musicki, Kramer et al. 2005; Yang and Ming 2006). The nNOS synthase is found in the cytosol of neuronal and skeletal muscle tissue and primarily functions in cell to cell communication while the iNOS is located in macrophages and within the cardiovascular system where cytokines and other inflammatory signals induce iNOS-dependent production of nitric oxide to defend against foreign pathogens (Sessa 1994). Researchers have shown that the NOS family can produce superoxide especially when lacking the substrate L-arginine (Xia, Dawson et al. 1996). The fact many NOS enzymes are localized near SOD enzymes and xanthine oxidases, as well as the inhibitory effect superoxide has been shown to have on nitric oxide production suggests the propensity for all ROS signaling to be co-dependent and tightly regulated by one another.

The xanthine oxidoreductase (XOR), first discovered as generator of endogenous superoxide in 1968 by McCord and Fridovich (McCord and Fridovich 1968), is made up of two forms of the same enzyme identified as xanthine oxidase and xanthine dehydrogenase. During purine degradation the oxidase form of the XOR enzyme produces both superoxide and nitric oxide species while the dehydrogenase form produces the potent antioxidant, uric acid (Hille and Nishino 1995). The XOR has therefore been considered a significant regulator of intracellular redox homeostasis and is widely used as generator of superoxide for *in vitro* experimentation (Afanas'ev 2005).

Peroxisomes are eukaryotic organelles responsible for both β-oxidation of lipids and the removal of damaged or obsolete proteins from the cytosol (Brown and Baker 2008). The peroxisomal fatty acyl-CoA oxidizing system utilizes oxygen and NAD as electron acceptors, but differs from mitochondrial β-oxidation by virtue of the reduction of molecular oxygen. Rather than creating superoxide or water, acyl-CoA oxidase transfers its electrons to oxygen, producing both *trans*-2-hexadecenoyl-CoA and hydrogen peroxide from palmitoyl-CoA (Osumi and Hashimoto 1978), similar to what has been observed for glyoxysomes, an organelle found in plants (Lazarow and De Duve 1976). To prevent the toxic build-up of hydrogen peroxide, peroxisomes contain high levels of the antioxidant enzyme catalase (Olsen 1998). Under normal physiological conditions the levels of hydrogen peroxide within the peroxisomes are minimized to prevent release into the cytosol. It is only when damaged or the amount of peroxisomal catalase is disrupted that hydrogen peroxide is released into the cytosol, causing oxidative stress and a variety of metabolic and neurological disorders (Sheikh, Pahan et al. 1998).

1.2.3 Antioxidants/enzymatic regulation

To maintain redox homeostasis and defend against the damaging aspects of ROS, a variety of enzymes exist to counteract the effects of ROS within the cell. To convert superoxide to the less reactive and more viable signaling molecule hydrogen peroxide two superoxide dismutase enzymes exist in mammalian cells, the cytoplasmic copper/zinc superoxide dismutase (Cu,Zn SOD) and the mitochondrial manganese superoxide dismutase (MnSOD). It is reasoned that mitochondria contain their own SOD because a significant amount of ROS production is associated with metabolic respiration (Paul, Belton et al. 2007). Further conversion of hydrogen peroxide to water and oxygen is mediated by the endogenous enzymes catalase. A variety of other molecules such as

ascorbic acid (Vitamin C), melatonin, uric acid, and tocopherols (Vitamin E) react non-enzymatically with ROS and thereby work in concert with other antioxidant enzymes to remove ROS as well as ROS-damaged substrates, such as oxidized lipids found throughout the cell (Cadenas 1997).

The antioxidant enzyme families, including systems, thioredoxins/thioredoxin reductase, peroxidases/sulfiredoxins, and the glutathione system (including the peptide glutathione/glutathione peroxidases/glutathione reductase/glutathione S-transferase) also are crucial for regulation of intracellular redox homeostasis. These enzyme systems function in pairs due to the mechanism by which they interact and regulate protein oxidation throughout the cell. This mechanism involves one or two reactive cysteines located within an active site that can interact with an oxidized protein thereby reducing that protein's thiols back to their sulfhydryl state (Holmgren 1995; Rhee, Kang et al. 1999). More specifically, the NH_3-terminal active site cysteine residue has a low pKa value and can therefore mediate a nucleophilic attack of the target disulfide to form a covalent mixed disulfide intermediate. This action is immediately followed by further reduction executed by the COOH-terminal active site thiolate, now deprotonated leaving a reduced target protein and active site disulfide in a thiol reducing enzyme such as thioredoxin (Berndt, Lillig et al. 2007). This sulfenic acid or disulfide is then reduced by the corresponding reductase partners, such as thioredoxin reductase using electrons from NADPH to continue functioning as reducing catalysts (Mustacich and Powis 2000). Overexpression of one or more of these antioxidants has been shown to prolong life span in several animal species from *C. elegans* (Fierro-Gonzalez, Gonzalez-Barrios et al.; Jee, Vanoaica et al. 2005; Olahova, Taylor et al. 2008) to mice (Weisbrot-Lefkowitz, Reuhl et al. 1998; Mitsui, Hamuro et al. 2002) further linking redox homeostasis to the aging process. Similarly, loss or mutation of one or more of these antioxidants often results in

heightened susceptibility to ROS-related disease states and aging (Kang, Rhee et al. 2005; Ballatori, Krance et al. 2009).

1.2.4 ROS-related disease states and ageing

1.2.4.1 ROS-related disease states

An increase in ROS has been implicated in a wide variety of pathological processes and can be sub-divided into two different categories. The first group is characterized by a shift towards oxidative stress and imbalance of the thiol/disulfide redox state of the cell, often resulting in damaged glucose tolerance leading to diabetes mellitus, carcinogenesis and a variety of neurological disorders (Ceriello and Motz 2004; Klaunig and Kamendulis 2004). It is believed that several oxidant generators including the mitochondria, NOX enzymes, and lipoxygenase produce ROS in response to hyperglycemic conditions associated with Type II diabetes (Brash 1999; Nishikawa, Edelstein et al. 2000; Li and Shah 2003). While ROS are not considered the outright cause of diabetes mellitus, its chronic production is thought to be involved in the consequences associated with diabetes, such as cardiovascular disease and retinopathy (Brownlee and Cerami 1981). Notably however, studies have also revealed that β-cells within the pancreatic islets have extremely low levels of antioxidant enzymes and this along with direct evidence in rats exhibiting β-cell vulnerability to hydrogen peroxide indicate these critical insulin producing cells are extremely prone to oxidative damage (Malaisse, Malaisse-Lagae et al. 1982). Antioxidant treatments demonstrate anti-apoptotic effects in β-cells and have exhibited beneficial effects in patients diagnosed with Type II diabetes by preserving the functioning β-cells left. More understanding, however, of the connection of ROS and the onset and progression of diabetes is needed.

While the link of ROS to carcinogenesis is well established the numerous roles ROS can play in tumor development and metastasis make it difficult to fully comprehend their mechanisms of action in every type of cancer. The major function attributed to ROS in the development of cancer is a result of the creation of DNA oxidative damage, leading to constitutive activation in proto-oncogenes such as c-Myc (Vafa, Wade et al. 2002) and Ras (Li, Firozi et al. 2002) as well as mutations in the tumor suppressor genes p53 (Hollstein, Sidransky et al. 1991) and $P16^{INK4a}$ (Hiroyasu, Ozeki et al. 2002) resulting in the loss of crucial cell cycle and DNA repair proteins and promotion of uncontrollable cell growth. While this initiation role of ROS in carcinogenesis remains true, the establishment of ROS as a signaling agent suggests aberrant ROS signaling cascades can also lead to alterations in normal regulatory processes and activation of transcription factors, such as AP-1 and NFκB, often leading to rampant cell growth (Klaunig and Kamendulis 2004). Disruptive ROS signaling, whether because of an increase in ROS production or a decrease in antioxidant enzymes, has been now been clearly demonstrated to play a role in carcinogenesis. It has been shown to lead to the upregulation of cellular processes such as angiogenesis which is considered a hallmark for tumor survival as well as the upregulation of certain genes such as those involved in mitochondrial biogenesis and glucose metabolism (Valko, Leibfritz et al. 2007).

ROS have also been implicated in several degenerative neurological disorders such as Alzheimer's and Parkinson's disease. As a result of the brain's high oxygen consumption and high concentrations of metal ions (Fe, Cu), it is considered extremely susceptible to oxidative damage over an individual's lifespan (Valko, Leibfritz et al. 2007). Mounting evidence suggests a direct relationship between oxidative stress and the development of Alzheimer's disease. Alzheimer patients display extensive oxidative damage in the form of lipid peroxidation (e.g. 4-hydroxynonenal), an increase in DNA

and protein oxidation including the crucial lipid transporter, apolipoprotein E (apoE), and an increase the damaging free radicals peroxynitrite and the hydroxyl radical (Butterfield, Castegna et al. 2002). Increased production of ROS has also been implicated in Parkinson's disease. Here abnormally functioning mitochondria, along with the presence of ferrous ions and superoxide lead to the oxidation of dopamine and production of the neurotoxic compound 6-hydroxydopamine. The presence of high amounts of 6-hydroxydopamine leads to the death of dopaminergic neurons within the midbrain and is considered a marker of Parkinson's disease (Jenner 2003). Similar to diabetes and carcinogenesis, it is difficult to separate and therefore study whether the role of ROS in these nueropathologies is the primary cause or a secondary consequence of other aberrant pathways already set in motion. The thiol antioxidants glutathione (GSH), N-acetyl-cysteine (NAC), and dithiothreitol (DTT), however, have been shown to protect dopaminergic neurons from dopamine autooxidation and apoptosis (Offen, Ziv et al. 1996) associated with Parkinson's disease suggesting modulation of redox poise could be a viable treatment option.

The second group of ROS-related diseases encapsulates other pro-oxidant inflammatory conditions and is characterized by an increase in ROS produced by the NOX family of enzymes as well as the XOR enzyme. These inflammatory oxidative conditions include atherosclerosis, ischemic/reperfusion injuries, and rheumatoid arthritis. An increase in ROS has been established to promote angiogenesis involved in the formation of atherosclerotic legions (Khatri, Johnson et al. 2004). This results from a variety of ROS-related effects such as oxidation of lipids, stimulation of VSMC proliferation and migration, and expression of adhesion molecules. The production of ROS involved in atherosclerosis primarily comes from the NOX family members found in the macrophages. These macrophages localize to inflamed regions within the blood

vessel, begin to accumulate low density lipids (LDL), and transform into adhesive foam cells that accumulate and obstruct the vessel (Ross 1999). The oxidation of LDL signals for further inflammation and compounds the injury. Antioxidant treatment has at times proven difficult possibly because of this overwhelming oxidative feedback loop, however, studies using the antioxidants probucol, a-tocopherol, and ascorbate have exhibited inhibition of atherosclerosis progression in animal models (Jialal and Devaraj 1996).

ROS generation resulting from ischemia/reperfusion injuries create serious complications for stroke victims and recipients of organ transplantation. During ischemia the XOR enzyme is primarily operating in its oxidase form. Once reperfusion finally occurs hypoxanthine and xanthine purine products that have accumulated are metabolized by the xanthine oxidase thereby generating massive amounts of superoxide and hydrogen peroxide (Granger, Stokes et al. 2001). This sudden and enormous amount of ROS production causes severe oxidative damage often signaling nearby cells to undergo apoptosis.

Chronic inflammation and oxidative injury are also associated with rheumatoid arthritis. This autoimmune disorder results in the accumulation of monocytes and lymphocytes within the synovial fluid. Once there the release of free radicals by the gathered phagocytes results in direct or indirect oxidative damage of nearby cartilage and extracellular matrix, as well as further aberrant ROS signaling, resulting in apoptosis or activation of metalloproteinases (Maurice, Nakamura et al. 1997). The synovial joints, like β-cells, contain low levels of the antioxidant SOD making them more vulnerable to oxidative damage (Mazzetti, Grigolo et al. 1996). Vitamin E has been demonstrated to reduce joint inflammation in transgenic KRN/NOD mouse model of rheumatoid arthritis (De Bandt, Grossin et al. 2002). The use of a superoxide dismutase mimetic (M40403)

also demonstrated relief of arthritic symptoms in a rat model of collagen induced arthritis (Salvemini, Mazzon et al. 2001) once again supporting a link between ROS and this disease state and providing viable treatment options.

1.2.4.2 ROS and the ageing process

Research utilizing the animal model *Caenorhabditis elegans* (*C. elegans*) has demonstrated that mutations in pathways which regulate oxidative stress often have an enormous impact on lifespan (Finkel, Serrano et al. 2007). In 1965, Denham Harman proposed the free radical theory of ageing (Harman 1956) stating the gradual decline in physiological function or ageing is the result of the accumulation of free radicals produced by metabolic respiration and the cumulative damage they do to DNA, lipids, and proteins within an organism over time (Hayflick 1998). This theory, along with others that have stemmed from it, is based on the fact that oxygen is a necessary evil in the survival of an organism (Balaban, Nemoto et al. 2005). A lack of oxygen would cause a halt to oxidative phosphorylation, a reduction in ATP, and cell death. During the process of aerobic metabolism in mitochondria, oxygen functions as a recipient of electrons that flux across the respiratory chain of redox donors and acceptors. Electrons, however, are prone to leaking even in healthy organisms (Hanukoglu, Rapoport et al. 1993; Salvador, Sousa et al. 2001). These electrons slip to molecular oxygen and generate the free radical/oxygen derivative superoxide. Approximate 1-3% of all molecular oxygen within the mitochondria of even healthy organisms is converted to superoxide (Benzi, Pastoris et al. 1992; Kinnula, Crapo et al. 1995). Many believe that while superoxide and other ROS can indiscriminately damage any macro-molecule in their proximity, it is the accumulation of superoxide-damaged mitochondrial DNA

(mtDNA) that eventually causes the mitochondria to shut down, energy production to cease, and the triggering of cell death that lead an organism to age (Cadenas and Davies 2000). A study of a mouse model containing a defective mitochondrial proof-reading polymerase resulted in an increase in mtDNA point mutations followed by the onset of a variety of age-related characteristics such as hair-loss, decreased fertility, and finally an overall decrease in lifespan compared to wild type (Trifunovic, Wredenberg et al. 2004). This evidence along with the confirmed role ROS plays in DNA damage places ROS as a major player the process of aging.

Studies on energy expenditure and lifespan in flies and worms reveal an inverse relationship between aerobic respiration/oxygen consumption and the lifespan of an organism (King and Barnett 1995; Gems and Doonan 2009). Also, an organism's rate of ROS production along with how effective its antioxidant defense system is has a dramatic impact on lifespan. Interestingly one of the most well known and controversial examples of the inverse relationship between oxygen consumption and lifespan in mammalian models, such as mice and rats, is the extended lifespan produced by caloric restriction. By reducing caloric intake, by approximately 30%, studies have shown a reduction in the generation of ROS and ROS-related DNA damage biomarkers such as 8-hydroxy-2'-deoxyguanosine (8-OH-dG) (Richter 1988; Barnett and King 1995). This reduction in both nuclear and mitochondrial DNA damage is believed to be directly related to the extension of lifespan and has since been adapted by some humans as a strategy to prolong life. Scientists are currently trying to uncover the regulatory pathways involved in the pronounced effect of caloric restriction on lifespan. One promising area of research involves the target of rapamycin (TOR) signaling pathway (for extensive review see (Sabatini 2006; Wullschleger, Loewith et al. 2006). The TOR signaling cascade has been established to regulate a variety of cell pathways in response to nutrient levels within the

cell. It is active when nutrient levels are high and promotes cell growth, and is inactive during periods of starvation. Many of the proteins involved in the TOR signaling pathway, such as PTEN, also represent well established targets of ROS signaling suggesting a potential cross talk between these two pathways.

Other research has uncovered an alternate theory by virtue of the function attributed to the uncoupling protein (UCP) family. UCPs have been shown upon activation to provide a pore within the ETC that allows protons to reenter the matrix from the intermembrane space. This not only lowers mitochondrial membrane potential and reduces the production of ATP by Complex V, but it is thought this also allows electrons to flux more efficiently across the ETC, thereby lowering electron slippage to oxygen and reducing ROS production (Korshunov, Skulachev et al. 1997; Liu 1999). The "uncoupling to survive" hypothesis therefore suggests the regulation of metabolic ROS production could be a built in mechanism for maintaining the same rate of oxygen consumption while uncoupling it from ATP production, thereby lowering the generation of ROS and preventing further damage to the cell (Brand 2000; Balaban, Nemoto et al. 2005). Studies in out bred mice revealed that those with the highest metabolic rates displayed longer life spans than mice with low metabolic rates. Mitochondria isolated from these mice revealed those with higher metabolic rates also exhibited and increase in proton conductance attributed to an increase in the activation of proton leaks through the adenine nucleotide transporter (ANT) and uncoupling protein 3 (UCP3) (Speakman, Talbot et al. 2004). The mechanism by which UCPs create a proton leak remains unknown and researchers are currently attempting to understand how these unique proton channels are involved in the regulation of redox homeostasis, metabolism, and overall cell fate decisions. It is not likely that ROS-related damage alone is responsible for the aging process, but rather the gradual malfunction and destabilization of a variety of

pathways within the cell and the mechanisms by which they communicate and regulate one another.

1.2.5 ROS-signaling

Emerging evidence implicates ROS as important mediators of signal transduction cascades involved in the regulation of cell fate. In order for a molecule to be considered a second messenger or signaling molecule, it must fall into four categories (Hancock, Desikan et al. 2001). First it must show substrate specificity and not indiscriminately modify anything in its path. While all ROS share the ability to react and damage DNA, proteins, and lipids within the cell, only hydrogen peroxide is thought to show the specificity necessary to be a suitable second messenger. The predominant target of direct oxidation by hydrogen peroxide is protein thiols, commonly referred to as reactive cysteines found in certain proteins within the cell.

The second criteria requires that the modification must be reversible, similar to an on and off switch. Proteins containing oxidized disulfide bridges or hydroxylated cysteines can be reduced back to sulfhydryl groups by antioxidant enzymes such as glutathione or thioredoxin.

Third, there must be some means of temporal regulation. The induction of the second messenger, in this case ROS, must be activated and operate according to physiological time scales. This includes the mitochondria and other oxidant generating systems. It is well established that several extracellular stimuli such as growth factors and cytokines as well as mitochondrial inhibitors and substrates can increase the production of ROS within the cell (Martindale and Holbrook 2002). The location of these oxidant generating enzymes and mitochondria as well as the cells ability to react to ROS inducing stimuli allow for the presence of ROS to be both spatial and temporal

thereby only being able to regulate signaling cascades at certain times and in certain places within the cell.

Lastly, there must be a system of enzymes capable of regulating the presence of the second messenger to prevent irreversible and unchecked alterations. This includes the well known antioxidant enzymes catalase and SODs as well as the antioxidant protein systems such as thioredoxin/thioredoxin reductase and glutathione/glutathione reductase which are capable of converting oxidized proteins back to their reduced states.

Cell Compartment	Biochemical Events	Cell response
Plasma Membrane /Cytosol	Inhibition of phosphatases; Activation of kinases: ERK, JNKs, p38, and the PI3/Akt pathway; activation of NF-κB	Mitogenesis Cell Survival Transcription of pro-inflammatory genes
Nucleus	Impaired DNA binding of AP-1, NF-κB, p53, and the Glucocorticoid receptor	Growth arrest Activation of the DNA damage response
Mitochondria	Mitochondrial damage activation of NF-κB and JNK-1 Hypoxic response Inhibition of proliferation signal transduction	Growth arrest Senescence Apoptosis Regulation of Metabolism

Table 2: Established ROS-signaling mechanisms in subcellular compartments

1.2.5.1 Mechanisms of redox regulation

One mechanism for direct regulation of a protein by ROS-signaling is through the oxidation of cysteine thiols. This either results in the creation of a disulfide bridge or a hydroxylated cysteine (*S*-hydroxylation) residue, both of which can be reduced back to sulfhydryls by the reducing catalysts thioredoxin or glutaredoxin. The acid constant (pKa) of most sulfhydryl groups is approximately 8.5-10, making them less vulnerable to

oxidation by hydrogen peroxide (Benesch, Lardy et al. 1955). In order for a cysteine to be considered oxidant sensitive, it must exist as a thiolate anion at a neutral pH (pH 7) (Filomeni, Rotilio et al. 2005). The presence of nearby basic amino acids, such as histidine, lysine, and arginine support this anion, allowing it to have a lowered pKa at a neutral pH (Copley, Novak et al. 2004). This is how most catalytic triads within the active sites of peroxidases and phosphatases work, and both of these enzyme families have been shown to have reactive cysteines prone to oxidation (Caselli, Marzocchini et al. 1998; Lee, Yang et al. 2002; Yang, Kang et al. 2002; Rhee, Kang et al. 2005). It is also possible for cysteines to become glutathiolated (S-glutathionylation). Glutathiolated proteins are present in minimal numbers under normal redox conditions, however their numbers increase significantly under oxidative stress conditions (Netto, de Oliveira et al. 2007). S-glutathionylation has hence been considered a protective modification in the presence of oxidative stress to prevent irreversible oxidation of cysteines to sulfinic (-SO2H) and sulfonic acids (SO3H) (Thomas, Poland et al. 1995). Likewise, it has also been well established that cysteines can be nitrosylated (S-nitrosylation) as a means of regulating cell function in response to nitric oxide signaling (Moncada and Palmer 1991; Hess, Matsumoto et al. 2005). The amino acid methionine also possesses a sulphur-containing side-chain susceptible to reversible hydroxylation important for signal transduction (Gao, Yin et al. 1998; Hoshi and Heinemann 2001).

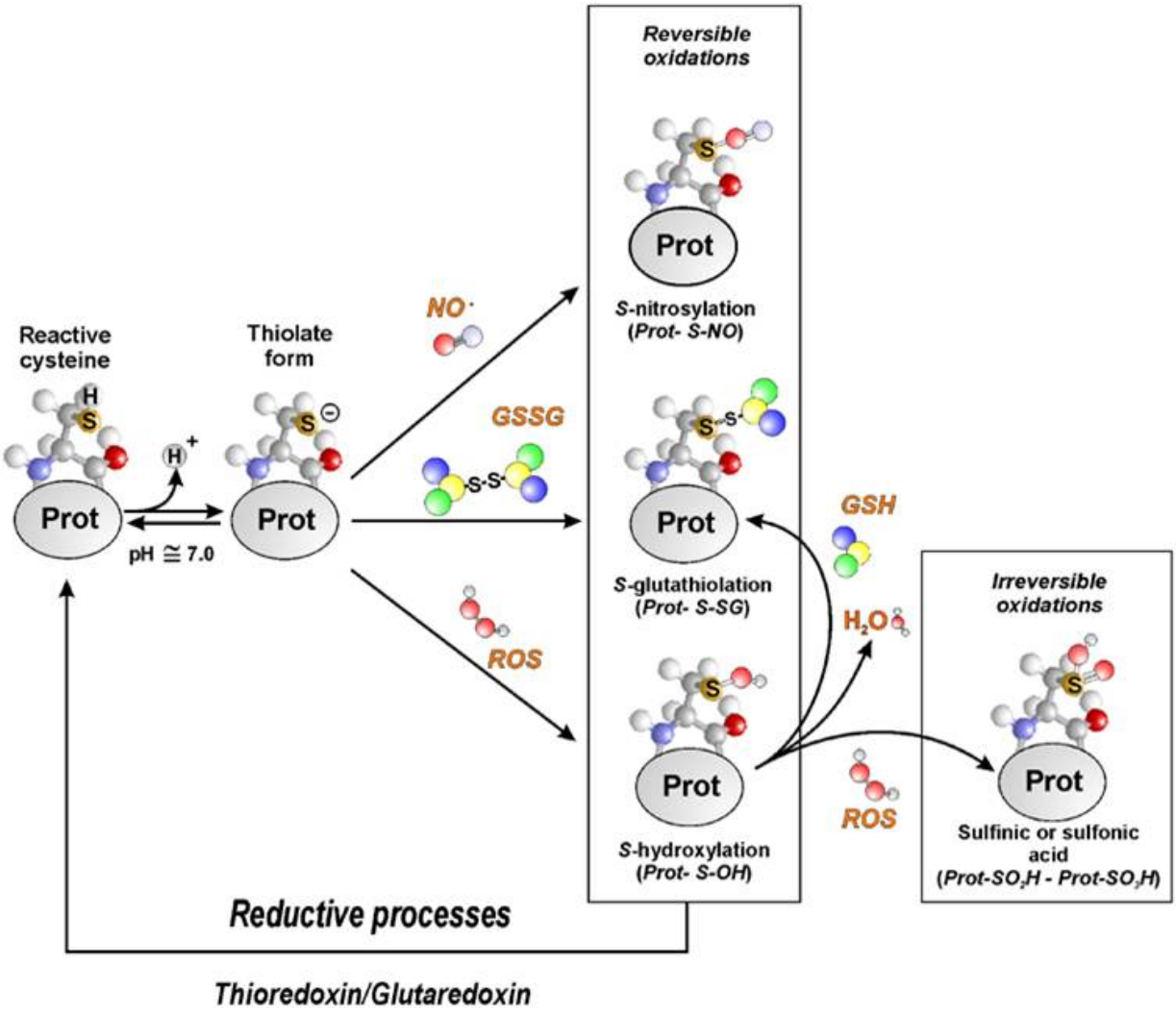


Illustration 1: Redox modifications of reactive cysteines

A reactive cysteine can exist as thiolate anion, under physiological pH. This allows them to more easily undergo reversible oxidation by either reaction with ROS (*S*-hydroxylation) or with the nitric oxide radical (*S*-nitrosylation). Reactive cysteines can also react with GSSG (*S*-glutathiolation). This last modification has been suggested to be a protective action by GSH to prevent the enzyme from further oxidations, which can lead to irreversible oxidation and protein damage. The thiol reducing enzymes thioredoxin and glutaredoxin bind to the oxidized cysteines and convert them back to their reduced sulfhydryl state. Image adapted from Filomeni, G et al 2005 Cell Death and Differentiation.

Proteins containing iron-sulfur centers can also be subject to redox regulation. Research demonstrates that either direct contact with superoxide or hydrogen peroxide or another oxidized substrate results in the release of free ferrous from the $[4Fe\text{-}4S]^{2+}$ cluster often acting as an off switch rendering those proteins inactive (Ramsay, Dreyer et al. 1981). This modification, however, is reversible and once the protein is no longer in close proximity with ROS the free ferrous group returns and the protein resumes its prior state of activity.

1.2.5.2 Downstream Targets

Not surprising given their reactivity and short half lives, one barrier towards understanding the functional roles of oxidants in cellular physiology is the identification of specific protein targets of oxidation *in vivo* (Janssen-Heininger, Mossman et al. 2008). Recent studies have successfully identified several cell signaling mediators sensitive to redox-regulation. As mentioned before, several phosphatases such as PTEN and protein tyrosine phosphatase (PTP) include a reactive cysteine in their active site that regulates their activity in response to mitogenic oxidant bursts (Lee, Kwon et al. 1998; Lee, Yang et al. 2002; Kwon, Lee et al. 2004). Mitogenic ROS bursts are therefore believed to activate mitogen-activated protein kinases (MAPKs) signaling pathways via the inhibition of phosphatases, thereby propagating phosphorylation cascades leading to the upregulation of genes involved in proliferation (Kolluri, Bruey-Sedano et al. 2003). Studies have also shown that a disturbance in the redox balance towards oxidative stress allows for the activation of stress-response kinases such as certain JNK isoforms that can then activate the transcription of antioxidant genes necessary to restore redox homeostasis (Han, Cao et al. 2006).

Thioredoxin and the peroxiredoxin family members represent antioxidant enzymes that contain either a redox-active dithiol or a reactive cysteine in their active sites. The 12 kDa thioredoxin is highly conserved from prokaryotes to eukaryotes and uses Cys32 and Cys35 within its active site to undergo reversible oxidation-reduction reactions (Holmgren 1985; Holmgren 1995; Nishiyama, Masutani et al. 2001). These redox active disulfide bridges allow thioredoxin to not only scavenger for free radicals but also bind and reduce a wide variety of oxidized proteins including the mammalian peroxiredoxins (Rhee, Kang et al. 1999). The ubiquitously expressed thioredoxin is a potent reducing catalyst vital to the maintenance of redox-homeostasis and the regulation of ROS-signaling.

A variety of transcription factors have also been shown to be direct targets of ROS signaling. OxyR in E. coli and Yap1 in yeast represent the prototypic oxidant sensing transcription factors. Each contains disulfide bonds that act as on/off switches, triggering the regulation of gene expression in response to oxidative stress (Zheng, Aslund et al. 1998; Wood, Storz et al. 2004). On the other hand, cysteine oxidation of the transcription factors p53, the p50 and p65 subunits of NF_KB and the c-Jun member of the AP-1 complex inhibits their ability to bind DNA and regulate gene transcription. In addition, the thiol reducing enzyme, thioredoxin 1 (Trx1) was found to increase p53-dependent transcription (Jayaraman, Murthy et al. 1997), to stimulate AP-1-driven transcription and DNA binding by forming a complex with directly oxidized Ref-1 (Hirota, Matsui et al. 1997), and to increase NF-κB-dependent transcription via reduction of the p50 subunit of NF-κB (Qin, Clore et al. 1995). Likewise, the ability of the glucocorticoid (GC) receptor, a transcription factor and closely related family member to NGFI-B, to bind to DNA and promote transcription is inhibited in the presence of ROS bursts and has been shown to directly interact with Trx1, implying that it is directly

oxidized. Hypoxia inducible factor 1 alpha (Hif1α) has also been shown to be directly nitrosylated (Sumbayev, Budde et al. 2003) and it is well-established that ROS stabilizes HIF1alpha under normoxic conditions (Metzen, Zhou et al. 2003).

Lastly there are also proteins, such as the mitochondrial enzyme aconitase, that contain iron/sulphur ($[4Fe\text{-}4S]^{2+}$) centers capable of redox regulation. In the presence of superoxide or hydrogen peroxide, the aconitase enzyme is rendered inactive as a result of the release of Fe-α in the ferrous oxidation state leaving aconitase with a $[3Fe\text{-}4S]^{1+}$ cluster (Ramsay, Dreyer et al. 1981). By virtue of the role mitochondrial aconitase plays in the conversion of citrate to isocitrate, it is thought by some that this process is an indicator of metabolic pathology and oxidative stress. However, because of the reversibility of this inactivation many now consider aconitase to be a potent redox regulator involved in a negative feedback loop (Bulteau, Ikeda-Saito et al. 2003). ROS inactivation of mitochondrial aconitase signals to slow the production of substrates for the electron transport chain thereby lowering the amount of mitochondrial ROS being produced. Once redox homeostasis has been resolved and the excess ROS has been cleared studies have demonstrated the free ferrous group returns to the aconitase enzyme thereby allowing it to resume its normal enzyme activity within the Krebs cycle (Ramsay, Dreyer et al. 1981).

1.3 NGFI-B

The immediate early gene-encoded orphan nuclear hormone receptor subfamily 4, group A, member 1, NR4A1 (NGFI-B / Nur77 / TR3) has pleiotropic functions in cell growth, metabolism, differentiation and death. Interestingly, NGFI-B is both induced and activated by stimuli / conditions that are highly coincident with increased ROS

production, including inflammation, ischemia, mitogenic growth factors, and fatty acid oxidation (Cheng, Volkers et al.; Zhao, Howatt et al.; Wu, Liu et al. 2002; Pei, Castrillo et al. 2005; Mullican, Zhang et al. 2007).

1.3.1 Nuclear Hormone Receptor Superfamily

Nuclear hormone receptors (NHRs) are a superfamily of structurally related transcription factors containing a highly conserved zinc-finger DNA binding domain (DBD) and less conserved ligand-binding domain (LBD), and a varying activation domain (AF). NHR involvement in the regulation of a variety of cell functions has been well described (Mangelsdorf, Thummel et al. 1995; Ribeiro, Kushner et al. 1995; Kumar and Thompson 1999; Aranda and Pascual 2001).

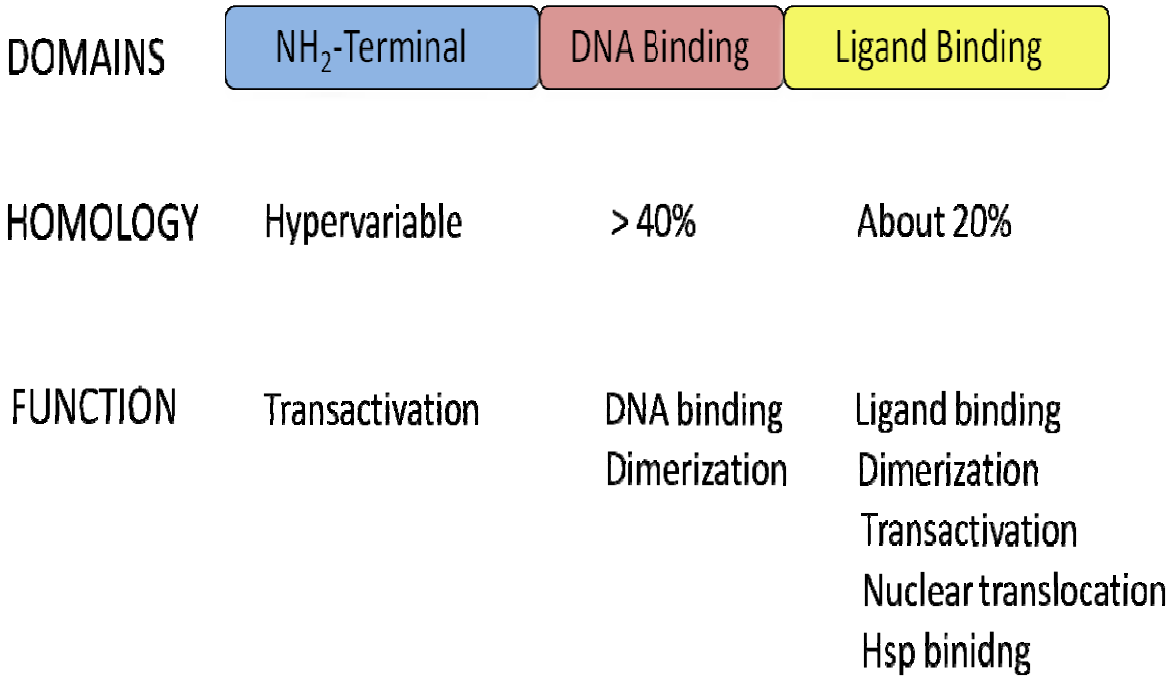


Illustration 2: Overall structure and domain homology of the NHR superfamily

The nuclear hormone receptor superfamily are characterized by an extremely homologous zinc finger DNA binding domain. The superfamily also contains a less homologous N-terminal transactivation domain and only slightly homologous C-terminal ligand-binding domain responsible for ligand binding, dimerization, and localization. Image adapted from Ribeiro, R et al 1995 Ann Rev Med.

NHRs were named for both their ability to bind the diverse hormones within the body as well as their prominent localization within the nucleus. The NHR family includes the better known estrogen receptor (ER), glucocorticoid (GC) receptor, the retinoic acid receptor (RAR), retinoid X receptor (RXR), and the liver X receptor (LXR). Once activated by their specific ligand nuclear hormone receptors bind as monomers, homodimers, or heterodimers to specific sequences within the DNA known as response elements (Aranda and Pascual 2001). Since the discovery of NHRs a sub-group of this transcription factor super family have been identified for which no ligand has been identified and appear to require no ligand activation. This subgroup is termed orphan NHRs.

1.3.2 NR4A subfamily

A chief role ascribed to the nuclear hormone receptor NGFI-B is as a transcription factor, binding to DNA, and regulating gene expression. When localized to the nucleus, NGFI-B, as well as Nurr1 and Nor1, can bind to the NGFI-B response elements (NBREs) as monomers or they can form homodimers and bind to the Nur77 response element (NuRE). Canonically, these palindromic binding sequences are AAAGGTCA (NBRE) and TGATATTTACCTCCAAATGCCA (NuRE). NGFI-B has also been shown to heterodimerize with RXR and bind to the DR5 response element (Wilson, Fahrner et al. 1991).

Similar to other steroid hormone receptors, the NR4A family (NR4A1-3) contains an N-terminal transactivation domain, a zinc finger DNA-binding domain, and a C-terminal ligand-binding domain (Hsu, Zhou et al. 2004).

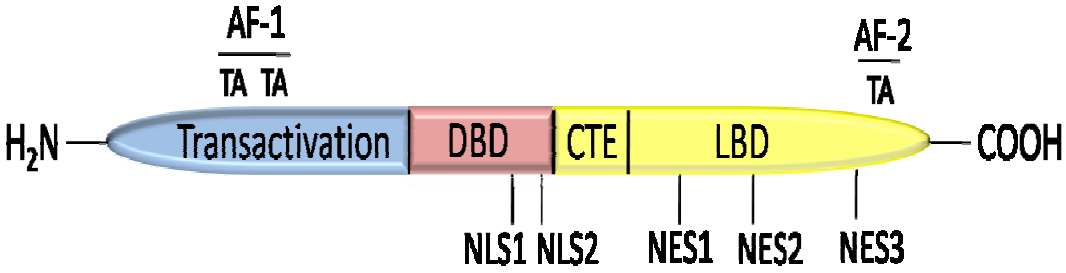


Illustration 3: NGFI-B Structure

NGFI-B contains an N-terminal transactivation domain (AF-1), a DNA binding domain (DBD) with two contiguous zinc finger DNA binding motifs, along with a ligand-binding domain (LBD) containing another ligand-independent AF-2 transactivation domain. The DBD contains two nuclear localization signals (NLS 1 and 2) while the LBD has been shown to have three different nuclear export signals (NES 1,2 and 3). Differences in post-translational phosphorylation near these NLS and NES regulate NGFI-B localization within the cell.

However, three-dimensional studies of the structure of NR4A1 (NGFI-B) and its closest relatives, NR4A2 (Nor1) and NR4A3 (Nurr1), have revealed that the NR4A proteins each contain an atypical ligand-binding domain with sterically bulky side-chains that preclude prototypical ligand-binding (e.g. steroids, fatty acids, retinoic acids, and other lipophillic molecules) (Wang, Benoit et al. 2003; Maxwell and Muscat 2006). Hence, the NR4A family members are classified as orphan nuclear hormone receptors because no physiological ligand has been identified that regulates their functions. Recently, Octaketide cytosporone B derived from the fungus *Dothiorella* was discovered as a naturally occurring agonist that binds directly to the NGFI-B ligand-binding domain, causing an increase in NGFI-B mediated transcription of genes involved in gluconeogenesis (Zhan, Du et al. 2008). Despite this and several other compounds, such as the purine anti-metabolite 6-Mercatopurine that indirectly activates NGFI-B the majority of evidence suggests the activity of the NR4A family members is mainly

controlled at the level of protein induction and posttranslational modification (Wansa, Harris et al. 2003; Pei, Castrillo et al. 2006).

1.3.3 Regulation of NGFI-B

1.3.3.1 Induction

The NR4A subfamily members are considered immediate early genes (IEGs) because of their rapid transcription in response to diverse physiological and physical stimuli, including growth factors, cytokines, fatty acids, phorbol esters, neurotransmitters, calcium, and shear stress (Maxwell and Muscat 2006). This ability to be rapidly induced in response to early changes in the cellular environment make the NR4A subfamily crucial in a wide variety of cellular processes. The fact that the NR4A family members are considered constitutively active once present makes regulation of their induction critical to cell fate determination.

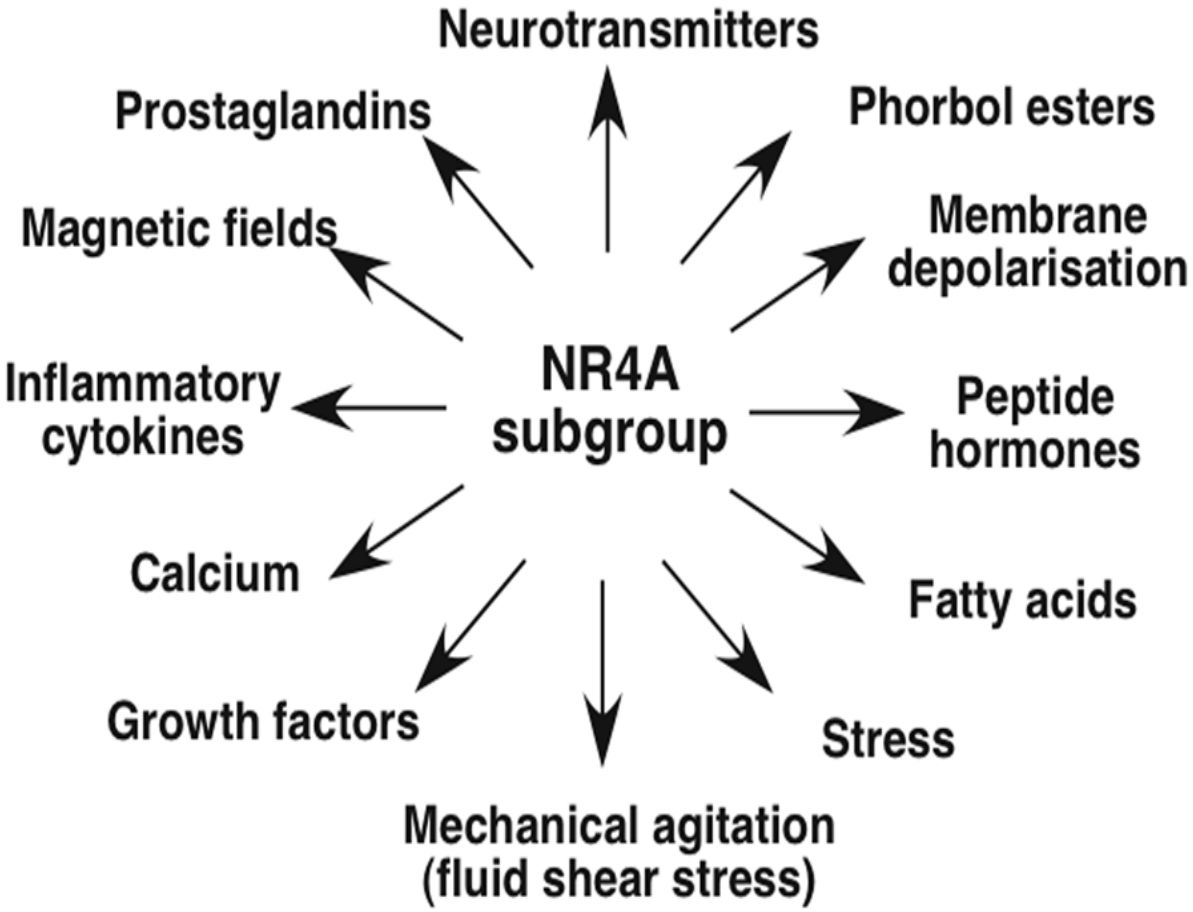


Illustration 4: Regulation of the expression of NR4A family members

NGFI-B and its close family members, Nurr1 and Nor-1, are rapidly induced by a wide variety of physiological and physical stimuli. Image adapted from Maxwell, M 2006 Nucl Recept Signal

1.3.3.2 Post-translational modifications

Numerous studies have illustrated that the key method for regulating the ligand-less, orphan nuclear hormone receptors is through post-translational modifications, primarily phosphorylation (Wingate, Campbell et al. 2006). These modifications affect DNA binding and cellular localization. Several kinases have been shown to phosphorylate and regulate NGFI-B localization and function, including Akt (PkB) kinase, c-Jun N-terminal kinase (JNK), and ERK2 or Mitogen Activated kinase 1 (MAPK1). In PC12 cells, an alteration in NGFI-B phosphorylation at Ser350 located within the "A-box" motif following treatment with nerve growth factor (NGF) abrogates NGFI-B DNA binding, thereby allowing the cells to differentiate (Katagiri, Hirata et al. 1997). Studies also reveal treatment with NGF results in the phosphorylation of ser105 of NGFI-B, bolstering an interaction with another NHR, retinoid X receptor (RXR), and causing their export from the nucleus to the cytosol mediated by the nuclear export signal (NES) of NGFI-B (Katagiri, Takeda et al. 2000). This NGFI-B-dependent redistribution of RXR decreases its ability to interact with the NHR, retinoic acid receptor (RAR), thus abrogating RXR-RAR, retinoid-dependent transcription. These modifications differ from that witnessed during membrane depolarization when PC12 cells are treated with KCl, resulting in NGFI-B binding and transactivation of NBRE (Katagiri, Hirata et al. 1997). Research also indicates that NGFI-B phosphorylation by either Akt or ERK2 in response to stimulation by growth factors results in the nuclear retention of NGFI-B and thus the promotion of proliferation and/or the prevention of cell death (Masuyama, Oishi et al. 2001; Pekarsky, Hallas et al. 2001). On the other hand, activation of MAP kinase kinase-1 (MEKK1) and JNK by various apoptotic stimuli bring about phosphorylation of NGFI-B resulting in its export from the nucleus and involvement in the induction of

apoptosis via the interaction of NGFI-B and Bcl-2 (Lin, Kolluri et al. 2004; Han, Cao et al. 2006).

1.3.4 Physiological functions of NGFI-B

1.3.4.1 Growth

Although NGFI-B is induced by various growth factors, it is not clear whether NGFI-B is directly involved in the promotion of cell growth. Evidence put forth by Kolluri et al. demonstrates NGFI-B nuclear localization and an increase NGFI-B-dependent transactivation upon treatment with epidermal growth factor (EGF) in lung cancer cells (Kolluri, Bruey-Sedano et al. 2003). Consistently, ectopic expression of NGFI-B promoted EGF-independent cell proliferation. Correspondingly, studies show exogenous expression of NGFI-B in human umbilical vein endothelial cells (HUVEC) promotes vascular endothelial growth factor A (VEGF-A) – independent proliferation, survival, and induction of several cell cycle genes (Zeng, Westermark et al. 2006). Studies have also revealed treatment with VEGF not only rapidly induces NGFI-B in human umbilical vein endothelial cells (HUVEC), but also reduces the ser351 phosphorylation known to reduce DNA binding and promote cytosolic translocation (Liu, Jia et al. 2003). While many of the downstream targets of NGFI-B-dependent transcription remain unknown, it is evident in response to certain growth factor signaling, NGFI-B nuclear retention and transcriptional activity promotes cell growth.

1.3.4.2 Cell Death

One of the initial functions attributed to NGFI-B was the regulation of T cell receptor mediated apoptosis (Woronicz, Calnan et al. 1994; Cheng, Chan et al. 1997). During the process

of negative selection, clonal deletions of self-reactive T cells are triggered to undergo programmed cell death. While some studies have confirmed that the inhibition of NGFI-B prevents T cells from undergoing apoptosis and the overexpression promotes T-cell apoptosis, other studies have illustrated compensation for the deletion of NGFI-B by the related family member, Nurr1 (Winoto and Littman 2002); (Liu, Smith et al. 1994); (Lee, Wesselschmidt et al. 1995). The mechanism for NGFI-B's role in T-cell receptor mediated apoptosis is attributed to its ability or inability to bind to DNA due to changes its phosphorylation state. The belief that nuclear localized NGFI-B dictates a cell's decision to proliferate or die was considered dogma until the discovery that NGFI-B could also localize to either the endoplasmic reticulum or the mitochondria and induce apoptosis (Rajpal, Cho et al. 2003). Interestingly, along with p53 and DJ-1/Park7, NGFI-B is one of only a few transcription factors established to localize in cytoplasmic, nuclear, and mitochondrial compartments (Hao, Giasson et al.; Springer and Kahle; Moll, Marchenko et al. 2006). The mitochondrial targeting of NGFI-B in certain types of cancer cells appears to regulate apoptosis (Li, Kolluri et al. 2000; Lin, Kolluri et al. 2004) This new localization was attributed not to the DNA binding domain of NGFI-B but to the ligand-binding domain despite lacking a mitochondrial localization sequence. The proposed method by which NGFI-B targets to the mitochondria involves interaction with the anti-apoptotic protein, Bcl-2. Further investigations have offered evidence that NGFI-B's interaction with Bcl-2 causes a conformational change, exposing the BH3 domain, and converting Bcl-2 from an anti-apoptotic protein to a pro-apoptotic protein.(Lin, Kolluri et al. 2004) (Kolluri, Zhu et al. 2008)

1.3.4.3 Differentiation

NGFI-B also accumulates in the cytosol in response to certain stimuli. There is evidence translocation to the cytosol is involved in driving the cell towards differentiation as well as mediation of the retinoid response.(Katagiri, Takeda et al. 2000) Several studies have demonstrated a unique regulation of NGFI-B in PC12 cells treated with nerve growth factor (NGF). Unlike other stimuli such as the membrane depolarizing agent, KCl, and epidermal growth factor (EGF), as mentioned above, NGF treatment inhibits transactivation of the NGFI-B response elements by causing the phosphorylation of serine-350, preventing DNA binding, and promoting the export of NGFI-B from the nucleus (Hirata, Kiuchi et al. 1993). Although the mechanism of NGFI-B regulation by NGF treatment is well described, the actual role NGFI-B plays in promotion of differentiation has not been well characterized. Recent studies of adipogenesis in NIH-3T3 adipocytes have contradicted one another over the role of the NR4A family in adipocyte differentiation; however, both agree NGFI-B is transiently induced in response to differentiation stimuli (Au, Payne et al. 2008) (Fumoto, Yamaguchi et al. 2007).

1.3.5 Disease states involving NGFI-B

NGFI-B is induced and activated in a wide array of tissues. As an IEG its presence at the protein level is transient often lasting between 3-6 hours. This rapid expression and degradation along with the tight regulation by post-translational modifications and cellular localization is necessary because of the constitutive activity of NGFI-B. Consequently, chronic expression of NGFI-B as result of the constant presence of stimuli known to induce NGFI-B often contributes to certain pathologies. For example, extensive investigation has revealed a role for NGFI-B in chronic inflammatory disorders such as rheumatoid arthritis. NGFI-B has long been established to play a crucial role in the regulation of genes involved in steroidogenesis and inflammation

(Crawford, Sadovsky et al. 1995; Fernandez, Brunel et al. 2000; Stocco, Lau et al. 2002) and its cytokine-dependent induction has been exhibited in rheumatoid arthritis synovial tissue (Murphy, McEvoy et al. 2001).

In the vessel wall, NGFI-B activation is associated with pathologic vascular remodeling associated with atherosclerotic legions in response to tumor necrosis factor-alpha (TNF-α), in part by inducing the plasminogen activator inhibitor-1 gene (PAI-1), ostensibly through an NGFI-B response element in the 5' upstream promoter region (Gruber, Hufnagl et al. 2003). NGFI-B is upregulated in macrophages stimulated by lipopolysaccharides (LPS), TNF-α, and phorbol 12-myristate 13-acetate and it is NGFI-B expressing lipid-loaded macrophages that have been discovered in atherosclerotic lesions (Barish, Downes et al. 2005; Pei, Castrillo et al. 2005).

Contrary to the inflammatory disorders associated with increased or prolonged expression of NGFI-B, carcinogenesis is considered a pathology that could in part result from the loss or inhibition of NGFI-B expression. Several cancer cell lines including colon, breast, prostate, and lung have demonstrated a role for NGFI-B in the induction of apoptosis by targeting to the mitochondria (Wu, Liu et al. 2002; Wilson, Arango et al. 2003; Lin, Kolluri et al. 2004; Lee, Ma et al. 2005; Thompson and Winoto 2008). This NGFI-B-dependent apoptotic response is attributed to its LBD which interacts with the anti-apoptotic protein, BcL-2 (Kolluri, Zhu et al. 2008). The interaction between NGFI-B and BcL-2 not only allows for mitochondrial targeting of NGFI-B despite not having a mitochondrial localization signal but also causes a conformational change in BcL-2, exposing its BH3 domain and likely preventing it from inhibiting pro-apoptotic proteins such as Bid and Bax (Li, Kolluri et al. 2000; Lin, Kolluri et al. 2004). Consistently, abrogation of NGFI-B and Nor-1 in mice led to fatal acute myeloid leukemia (AML),

suggesting a role for the NR4A family as potential tumor suppressors (Mullican, Zhang et al. 2007).

As a result of the pleiotropic nature of NGFI-B it is difficult to predict whether its prolonged presence or inhibition is going to instigate pathological signaling cascades in various tissues. Further investigation is needed to understand the delicate balance associated with NGFI-B activity as well as better comprehension of how NGFI-B is regulated once present on the protein level in order to combat the diverse NGFI-B-dependent disease states.

1.4 MITOCHONDRIAL BIOENERGETICS

1.4.1 The Mitochondria

Often referred to as the "powerhouse" of the cell, the mitochondrion is a unique membrane-bound organelle found in varying numbers in most eukaryotic cells and is responsible for the production of chemical energy in the form of adenosine triphosphate (ATP). Mitochondria are made up of specialized compartments that allow them to carry-out its crucial function in energy production. These sections are the intermembrane space and the matrix and are divided by from the cytoplasm by distinct double-phospholipid membranes known as the outer membrane (OM) and the inner membrane (IM). The cristae are structures formed by the unique folding of the IM and a variety of membrane bound enzymes, including members of the electron transport chain (ETC) reside here. This folding increases the surface area and allows for the maximal production of ATP per mitochondria.

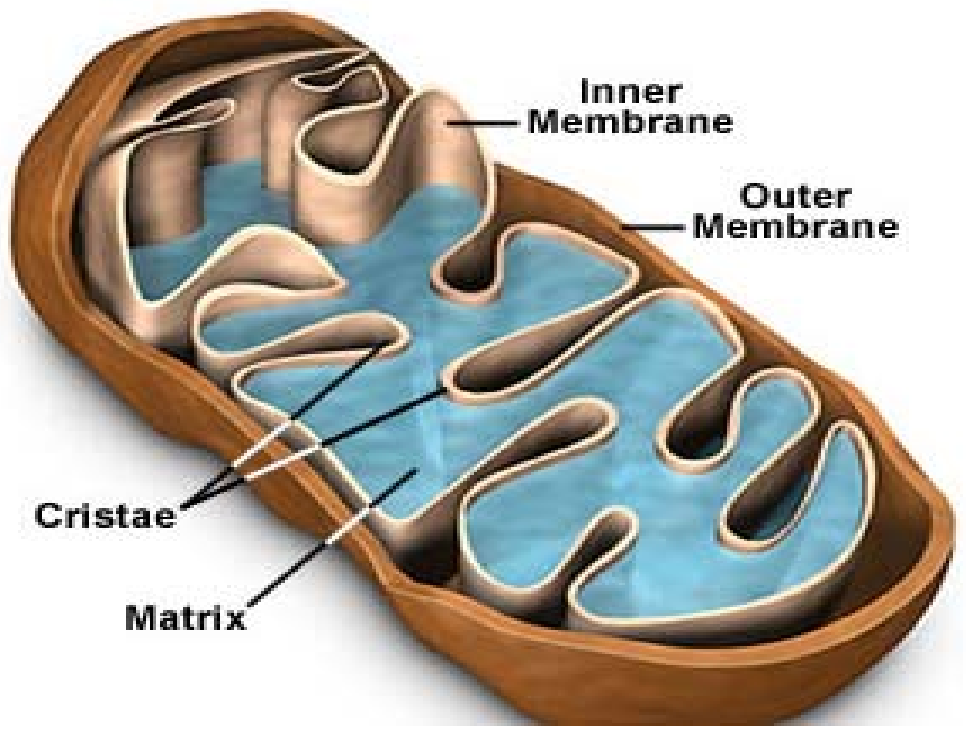


Illustration 5: Mitochondria structural features

Mitochondria are made up of specialized compartments that allow them to carry-out its crucial function in energy production. These sections are the intermembrane space and the matrix and are made possible due to distinct double-phospholipid membranes known as the outer membrane (OM) and the inner membrane (IM). The cristae are structures formed by the unique folding of the IM
Image adapted from: http://micro.magnet.fsu.edu/cells/mitochondria/mitochondria.html

Mitochondria are believed to have originated from prokaryotic bacteria that were incorporated into the cell through endocytosis from which a symbiotic relationship was established (Sagan 1967). The incorporation of these bacteria with respiratory abilities would have offered an evolutionary advantage to an organism solely producing energy from either glycolysis or fermentation (Bruce Alberts 2007). Mitochondria contain their own DNA that is in a circular shape, similar to that found in bacteria. This mitochondrial DNA (mtDNA) codes for some components of the respiratory complexes as wells as the ribosomes and other machinery necessary for their translation and upkeep.

Despite the advantage mitochondria afford thanks to the massive amounts of chemical energy they produce, mitochondrial respiration is considered the main producer of ROS and therefore one of the main contributors to the ageing process and other ROS-related disease states. In this way, the gift of large amounts of energy production is offset by the introduction of ticking clock. However, with the recent evidence that ROS are also crucial signaling molecules, the formation of a whole new outlook has begun concerning the role mitochondria play within cells. More and more studies continue to uncover new regulatory roles for the mitochondria and further investigation may uncover an exciting link between several cellular signal transduction pathways and the products produced by the mitochondria.

1.4.2 The Electron Transport Chain (ETC)

The ETC consists of a series of electron donors and acceptors within the mitochondria of eukaryotic cells. Products generated by the Krebs cycle within the mitochondrial matrix, NADH and succinate $FADH_2$, donate electrons to NADH coenzyme Q reductase (Complex I) and succinate dehydrogenase (Complex II),

respectively. These electrons continue to be passed along the ETC from donor to a more electronegative acceptor until they are delivered to the most electronegative acceptor, oxygen thereby reducing oxygen to water. Once electrons have been delivered to Complex I and II, these complexes each donates its electrons to coenzyme Q. Coenzyme Q passes electrons to the cytochrome bc_1 complex (Complex III) which then donates electrons to cytochrome c. Electrons are finally passed from cytochrome c to cytochrome c oxidase (Complex IV), which reduces molecular oxygen to water.

This flux of electrons through the chain generates energy and causes conformational changes allowing for proton pumping (four from Complexes I and IV and six from Complex III) from the matrix to the intermembrane space. This creates an electrochemical proton gradient known as the mitochondrial membrane potential (MMP). The matrix, containing fewer protons than the intermembrane space, is more electro-negative therefore given the opportunity protons will naturally want to flow back into it. The lipid bi-layer making up the intermembrane maintains this membrane potential leaving only the F1/F0 ATPase synthase as a means for protons to flow back through to the matrix and resulting in the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP).

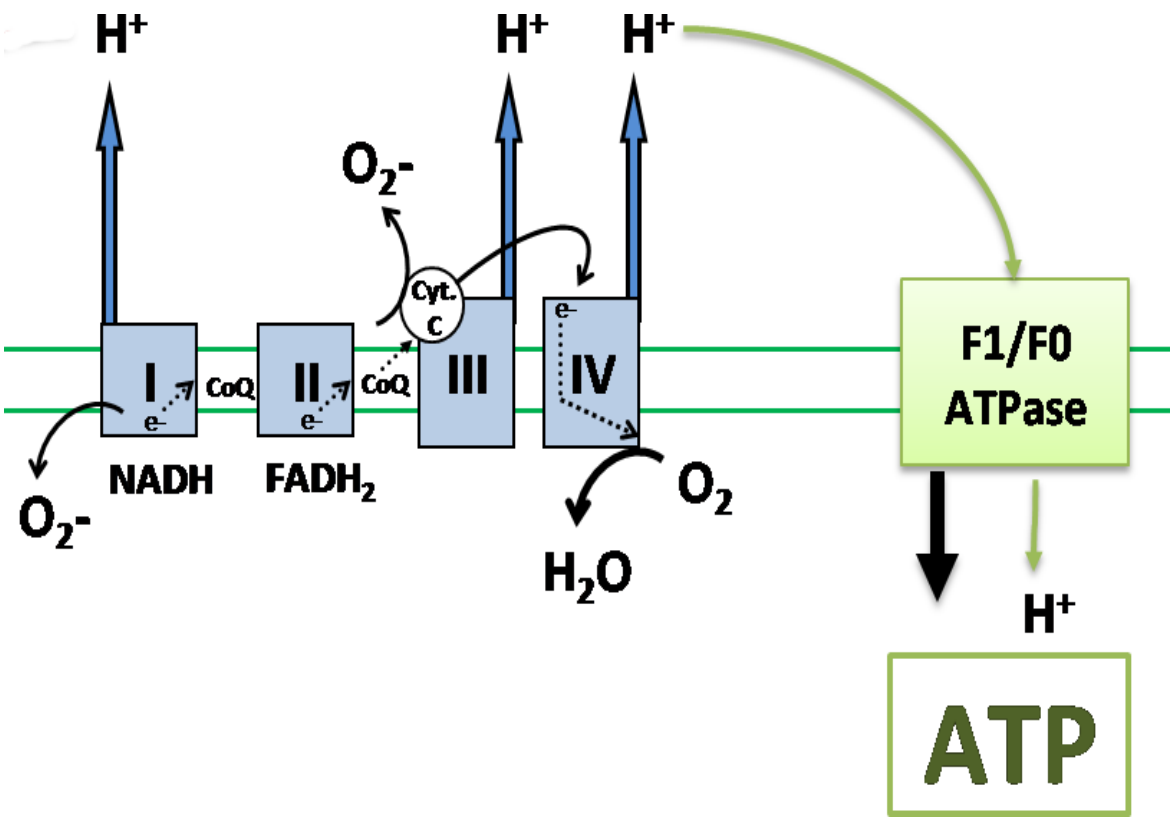


Illustration 6: The mitochondrial electron transport chain

The ETC consists of a series of electron donors and acceptors within the mitochondria. Products generated by the Krebs cycle within the mitochondrial matrix, NADH and $FADH_2$, donate electrons to NADH coenzyme Q reductase (Complex I) and succinate dehydrogenase (Complex II), respectively. These electrons continue to be passed along the ETC from donor to a more electronegative acceptor until they are delivered to the most electronegative acceptor, oxygen thereby reducing oxygen to water. This flux of electrons through the chain generates energy and causes conformational changes allowing for proton pumping from the matrix to the intermembrane space creating an electrochemical proton gradient. This electrochemical gradient provides the driving force for the production of ATP from ADP by the F1/F0 ATPase synthase.

1.4.2 The Mitochondrial Membrane Potential (MMP)

The mitochondrial membrane potential is the voltage difference between the mitochondrial intermembrane space and the matrix. Nobel Laureate, Peter Mitchell discovered the mitochondrial membrane potential is the driving force for the generation of energy in the form of ATP (Mitchell 1966; Mitchell 1979). NADH and $FADH_2$ are capable of donating their electrons to Complex I or Complex II, respectively. This reaction is coupled to the pumping of protons from the matrix to the intermembrane space. The accumulation of protons in the intermembrane space gives it a positive charge compared to the matrix creating an electrochemical gradient that drives protons through the ATPase thereby generating ATP (Mitchell 1979). So in order for the mitochondria to produce ATP efficiently and the cell to remain viable it is important to maintain this membrane potential.

1.4.2.1 Regulators of MMP

As other roles of the mitochondria within the cell have been discovered the mitochondria membrane potential has become an important indicator of mitochondrial functions such as apoptosis and ROS production. Still, controversy exists regarding the different mechanisms by which the MMP is regulated. One theory involves the mitochondrial permeability transition pore (MPTP). The voltage-dependent anion channels (VDAC), adenine nucleotide translocase (ANT), and cyclophillin d combine to form what is commonly referred to as the MPTP (Halestrap, McStay et al. 2002). The pore exists in three main positions. The MPTP can be found in a closed position, characterized by an intact MMP and functioning oxidative phosphorylation. Partial opening of the MPTP or “low conductance” allows for the influx of ions 300 Daltons or

less and the reversible loss of the MMP. "High conductance" or full opening of the MPTP is associated with an irreversible loss of MMP. Complete opening of the MPTP leads to an increase in mitochondrial permeability to solutes less than 1.5 kDa (Ichas and Mazat 1998; Gottlieb 2001; Ly, Grubb et al. 2003). Studies have shown that alterations in either cytoplasmic or mitochondrial calcium concentrations can trigger the pore to open and cause temporary loss of membrane potential (Gunter, Buntinas et al. 2000). Research also suggests that the regulation of MPTP opening is crucial to protecting tissue from the excessive oxidative stress that is associated with ischemia-reperfusion injury (Saotome, Katoh et al. 2009).

Others suggest the opening of the MPTP allows for the release of apoptotic factors, including cytochrome c, apoptosis inducing factor (AIF), and Smac/Diablo, from the mitochondria (Smaili, Hsu et al. 2000). Disagreement exists concerning the mechanism by which the MPTP regulates apoptosis. It is believed by some that opening of the MPTP results in the influx of ions such as potassium, magnesium, and calcium, as well as water (Bernardi, Vassanelli et al. 1992). This causes the mitochondria to swell and the outer mitochondrial membrane to burst (Crompton 1999). Studies using the cyclophilin d inhibitor cyclosporin A (CspA) appears to block apoptosis in some cell lines, lending strength to this particular theory (Petronilli, Cola et al. 1993). However, other research has shown circumstances in which the MPTP remained closed and the MPP intact despite cytochrome c release and caspase activation (Bossy-Wetzel, Newmeyer et al. 1998). Controversy still surrounds the exact role the MPTP has in mitochondrial function. A variety of theories exist concerning the involvement of the MPTP in regulating cellular calcium, the release of apoptotic factors, as well as the regulation of mitochondrial ROS production. Evidence suggests that it is likely involved in all of these processes, therefore to further understand these possibilities more research

is needed to define the mechanism by which the MPTP opens and closes and its regulation by specific factors.

Uncoupling proteins (UCPs) are intermembrane proteins located within the mitochondrial inner membrane also known to lower MMP. The involvement of the first uncoupling protein, aptly named UCP1, in cold-induced thermogenesis in mammalian brown adipose tissue has been well established (Girardier and Schneider-Picard 1983; Cannon and Nedergaard 2004). Research has demonstrated that upon activation UCP1, and its close homologues UCP2 and 3, leak protons from the intermembrane space back into the mitochondrial matrix (Mills, Banks et al. 2003; Esteves and Brand 2005). This UCP-dependent proton conductance uncouples electron transport from the oxidative phosphorylation of ADP to ATP releasing the energy lost as heat resulting in an increase in oxygen consumption and the decrease of the MMP (for a comprehensive review see (Garlid, Jaburek et al. 2001; Brand and Esteves 2005; Krauss, Zhang et al. 2005). The lowered MMP, allows electrons to flux more efficiently through the ETC, thereby preventing the slippage of electrons at Complex I and III decreasing the generation of superoxide (Brand, Affourtit et al. 2004; Brand, Buckingham et al. 2004). The ability of the mitochondria to regulate how much ROS is produced suggests that the mitochondria could be actively involved in cellular redox-signaling, and that the MMP could be used as an indicator of this involvement.

Loss of MMP has also been associated with the intrinsic apoptotic pathway. Pro-apoptotic members of the Bcl-2 family including Bid, Bak, and Bax are thought to allow for Bax homo-oligomerization which creates pores within the outer membrane (Silke and Vaux 1998; Korsmeyer, Wei et al. 2000; Yin 2000). This allows for the release of pro-apoptotic proteins from the mitochondria, such as cytochrome c, and leads to the activation of caspase-9 and the formation of a multimeric apoptosome complex (Zou, Li

et al. 1999). Eventually, caspase-3 is cleaved resulting in the further propagation of cell death (Zou, Li et al. 1999; Acehan, Jiang et al. 2002). These pores not only allow mitochondrial proteins to enter the cytosol, but also allow the release of protons into the cytosol, triggering a loss of membrane potential. However, as a result of the rapid nature of programmed cell death it is unclear whether regulation of membrane potential is necessary for programmed cell death or merely an artifact. Studies have also indicated that upon activation, caspases enter the mitochondria and cleave the p75 subunit of respiratory Complex I rendering it inactive (Ricci, Munoz-Pinedo et al. 2004). This results in a reduction in protons being pumped into the intermembrane space thus lowering the MMP by an entirely different mechanism.

1.5 SIGNIFICANCE OF STUDYING THE REDOX REGULATION OF NGFI-B

As reviewed by Maxwell *et. al.*, (Nuclear Receptor Signaling, 2005), NGFI-B responds to diverse stimuli that are also associated with increased production of cellular ROS, including prostaglandins, phorbol esters, fatty acids, cell stress, mechanical agitation, mitogenic growth factors, and inflammatory cytokines. Though its physiologic functions are far from fully established, NGFI-B is involved in dopaminergic neuronal development, steroidogenesis, T-cell selection (apoptosis), and adipose development. Arguably, the most important disease association for NGFI-B is atherosclerosis, which is strongly linked to oxidative stress and vascular remodeling involving circulating macrophages, and vessel wall endothelial and smooth muscle cells (Zafari, Ushio-Fukai et al. 1998). In the vessel wall, NGFI-B activation is associated with pathologic vascular remodeling in response to tumor necrosis factor-1 alpha (TNF-α). This NGFI-B activation results in the downstream induction of the plasminogen activator inhibitor-1

(PAI-1) gene, ostensibly through an NGFI-B response element in the 5' upstream promoter region (Gruber, Hufnagl et al. 2003). Similarly, TNF-α is well known to increase endothelial ROS production; and increased vascular oxidative stress and PAI-1 levels each is associated with vascular disease risk (Li and Fukagawa). NGFI-B induction occurs in response to smooth muscle cell proliferative stimuli as well as low concentrations of exogenous hydrogen peroxide. Thus, reductive inhibition of NGFI-B in the vasculature may comprise a new therapeutic avenue for the prevention of neointimal formation, and plaque development during atherogenesis.

It is also well established that oxidative stress and dependence on both mitogenic and anti-apoptotic ROS signaling in cancer cells represent specific vulnerabilities that can be selectively targeted. This new form of chemotherapy, termed redox chemotherapy, utilizes direct- or indirect-acting redox modulators as adjuncts to other chemotherapeutic drugs in order to better target and treat certain types of cancer (for a comprehensive review (Wondrak 2009). In a wide variety of cancer cells lines the translocation of NGFI-B to the mitochondria in response to apoptotic stimuli has been observed (Li, Kolluri et al. 2000; Wu, Liu et al. 2002; Lin, Kolluri et al. 2004; Lee, Cobb et al. 2007; Thompson and Winoto 2008). This new localization was not dependent on its DNA binding domain, but rather to the ligand-binding domain despite lacking a mitochondrial localization sequence. NGFI-B induction occurs after treatment with the tumor promoter and ROS generator, TPA and studies in cardiomyocytes have revealed mitochondrial localization by NGFI-B under oxidative stress (Cheng, Volkers et al.). These findings along with the discovery that gene knockouts of NGFI-B and Nor-1 lead to acute myeloid leukemia in mice (Mullican, Zhang et al. 2007) suggest further understanding of the redox regulation of NGFI-B is necessary and could lead to the development of potential therapeutic strategies in the treatment of cancer.

Long term exposure of dopaminergic neurons to pro-oxidant conditions increases their susceptibility to apoptosis and this exposure is suspected to be associated with the neurological diseases such as Parkinson's disease (No, Bang et al.; Kulich and Chu 2003). Whether oxidants are a cause or the consequence of neuropathologies, such as Parkinson's, remains unclear. However, the association of increased oxidant production with dopamine metabolism suggests their role is worth further investigation. Interestingly, the close family member of NGFI-B, Nurr1, is not only shown to be expressed in the central nervous system, including the substantia nigra, but knock down of Nurr1 results in increased apoptosis in the midbrain dopaminergic neurons known to deteriorate in Parkinson's disease (Rhee, Chang et al. 2003; Jankovic, Chen et al. 2005). Enhanced comprehension of how NGFI-B and the other NR4A family members respond to ROS signaling will possibly lead to better treatments for and understanding of neuropathologies such as Parkinson's and Alzheimer's disease. The results put forth in this body of work provide a new biochemical framework to begin to address potential mechanisms linking NGFI-B and cellular redox changes to similarly pleiotropic physiological and pathological processes.

1.6 SIGNIFICANCE OF NGFI-B REGULATED MMP LOSS IN SKELETAL MUSCLE CELLS

NGFI-B was characterized initially for its involvement in T-cell selection through apoptosis. The role of NGFI-B in the apoptotic process was originally believed to involve nuclear localization NGFI-B-dependent transcription. Consistent with this idea, overexpression of a dominant-negative NGFI-B protein that inhibited the activity of all three NR4A family members prevented T-cell negative selection (apoptosis) (Calnan, Szychowski et al. 1995; Zhou, Cheng et al. 1996). Additionally, several downstream

targets of NGFI-B-dependent transcription such as TRAIL, Fas ligand, and NDG1 are associated with the activation of apoptosis through caspase-8 and the death receptor pathway (Rajpal, Cho et al. 2003). This evidence along with experiments showing that both constitutive expression of NGFI-B as well as transient expression of a mutant form of NGFI-B with higher transcriptional activity lead to massive apoptosis in thymocytes highly suggests that NGFI-B-dependent transcription is likely at the root of its apoptotic function (Cheng, Chan et al. 1997; Kuang, Cado et al. 1999). However, studies using a dominant negative form of the protein FADD that blocks death receptor signaling revealed thymocytes still underwent negative selection implying other apoptotic mechanisms may be at play, including the translocation of NGFI-B to the mitochondria (Newton, Harris et al. 1998).

More recently, evidence in not only thymocytes, but a variety of cancer cell lines has indicated a mitochondrial localization of NGFI-B in response to a variety of apoptotic stimuli that appears to promote cell death (Li, Kolluri et al. 2000; Wu, Liu et al. 2002; Wilson, Arango et al. 2003; Lin, Kolluri et al. 2004; Lee, Ma et al. 2005; Thompson and Winoto 2008). Zhang et al. have shown that NGFI-B requires an interaction with Bcl-2 in order to induce apoptosis in prostate cancer cells (Li, Kolluri et al. 2000; Lin, Kolluri et al. 2004). In their reports, the ligand-binding domain of NGFI-B causes a conformational change in Bcl-2 that converts it to a pro-apoptotic protein by exposing its BH3 domain (Lin, Kolluri et al. 2004). Numerous other publications corroborate NGFI-B mitochondrial localization and the regulation of the BH3-exposed Bcl-2 in apoptosis in a variety of cells lines. However, the physiological relevance and overall mechanism by which NGFI-B activates programmed cell death remains largely unknown.

The discovery that both transient and stable expression of NGFI-B in L6 skeletal muscle cells resulted in the loss of MMP in the absence of any apoptotic stimuli led us to

hypothesize that this could possibly be an early event in NGFI-B-dependent cell death induction. Muscle cells, like neuronal cells, are resistant to cell death compared to other cell types, especially once they have differentiated (Wang and Walsh 1996; Kamradt, Chen et al. 2002). This resistance to cell death is crucial because skeletal muscle is a primary site of fatty acid oxidation and glucose disposal and largely involved in insulin sensitivity and obesity (Maxwell, Cleasby et al. 2005; Chao, Zhang et al. 2007). Muscle fibers being post-mitotic ensure that once lost they can never be regained therefore resulting in the total loss of their functional outputs. NGFI-B has been previously described by George Muscat's lab to be upregulated in C2C12 muscle cells by the β-adrenergic receptor (β-AR) agonist isoprenaline. Using siRNA mediated knockdown of NGFI-B, several genes involved in energy expenditure and lipolysis were downregulated, including the uncoupling proteins UCP2 and UCP3 (Maxwell, M 2005 JBC). Likewise, NGFI-B induction by β-AR stimulation was demonstrated and linked to the induction of the uncoupling protein, UCP1, in brown adipocytes of cold-induced mice (Kanzleiter, Schneider et al. 2005). This evidence not only suggests cross-talk between the β-AR and nuclear hormone receptor signaling but also provides a possible mechanism by which NGFI-B indirectly lowers MMP through the upregulation of uncoupling proteins. However, oxygen consumption experiments using stable L6 muscle cells, expressing either empty vector or NGFI-B, revealed a significant difference in State 3 respiration but no differences in State 4 respiration. This suggests that the NGFI-B-dependent alteration of MMP is not caused by proton leak. Further, systematic, exploration into how NGFI-B is regulating the MMP will lead to a better understanding of how mitochondria regulate signal transduction pathways and may reveal a mechanism for NGFI-B-dependent cell death induction.

1.7 DISSERTATION OBJECTIVES

The primary focus of my work was to characterize a unique redox signaling pathway involving the orphan nuclear hormone receptor NGFI-B. I initially approached this objective by demonstrating that NGFI-B-mediated transcription plays a novel role as a sensor coordinating cellular redox status and the regulation of gene expression. Notably, both exogenous oxidants and physiologically-relevant NGFI-B-inducing stimuli can stimulate NGFI-B induction and activity in multiple cell types. Correspondingly, experiments also demonstrate that NGFI-B-dependent transcription is potently inhibited by thiol-reducing agents through a novel interaction between NGFI-B and the cellular thiol reducing enzyme thioredoxin 1 (Trx1). Together, these studies reveal a hitherto unappreciated role for NGFI-B as a mediator of a redox-sensitive pathway of gene regulation. Moreover, the studies add NGFI-B to an expanding portfolio of potential substrates for Trx1, and shed light on a novel, inhibitory function of Trx1 in the regulation of gene expression. This work lays the foundation for future work aimed at understanding in more detail the direct and/or indirect biochemical mechanisms underlying the redox regulation of NGFI-B in more detail.

The second part of my work stemmed from a unique discovery that stable expression of NGFI-B in L6 skeletal myoblasts resulted in the loss of MMP. Further characterization of this finding indicated that while these cells basally expressed full-length NGFI-B they did not appear to undergo apoptosis. The loss of MMP often preceeds apoptosis and signifies damage to or disruption of the outer mitochondrial membrane. It is unclear, however, if the loss of MMP is a necessary step in the apoptotic process or simply a consequence of the intrinsic apoptosis pathway. On one hand, it makes sense that the MMP would be lost upon outer membrane disruption. However, very little is still know regarding the maintenance of the MMP, and a variety of other

factors, including the MPTP and uncoupling proteins, have been shown to temporarily abrogate the MMP for reasons other than cell death induction. Further investigation into the mechanism by which NGFI-B regulates the MMP in L6 muscle cells could advance our understanding of mitochondrial-dependent cell signaling and apoptosis.

Chapter 2: Methods and materials

2.1 Methods and materials for Chapter 3

2.1.1 Plasmid DNA constructs

The full-length coding sequence of mouse NGFI-B was sub-cloned into the pcDNA6/myc-His vector (Invitrogen) in using EcoRI and XhoI. Full-length human thioredoxin1 was sub-cloned using BamHI and XbaI into pcDNA3.1/V5-His (Invitrogen). The NBRE luciferase reporter construct (JA982) containing three consecutive NBRE sequences upstream of the luciferase gene was a gift from Jacques Drouin, University of Montreal, Montreal, Canada. The ΔAF-1-NGFI-B-myc construct was generously provided by Justin Rochford, University of Cambridge, United Kingdom.

2.1.2 Cell Culture and Transfection Assays

PC12 rat pheochromocytoma cells grown on collagen type IV coated plates/flasks in DMEM (Mediatech) growth medium containing 10% fetalplex serum (Gemini) and 1% penicillin/streptomycin (Gibco). PC12 cells were grown in collagen coated flasks to improve their adherence. The culture medium was changed every 2 days. Rat vascular smooth muscle cells (VSMC) and Hek293T cells were cultured in DMEM growth medium containing 10% fetal bovine serum and 1% penicillin/streptomycin.

2.1.3 Immunoblotting

Lysates were prepared in RIPA buffer (50mM Tris-HCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150mM sodium chloride (NaCl),

2mM EDTA, pH 8.0). A bicinchoninic assay (Pierce Biotechnology) was used to quantify total protein concentrations. Samples were prepared with 4X sample buffer (250mM Tris-HCl, 8% SDS, 40% glycerol, 8% betamercaptoethanol, 0.02% bromophenol blue) and separated by electrophoresis on 12.5% polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane and probed with primary antibody according to the manufacturer's instructions. Antibodies used were mouse monoclonal 2E1 antibody specific for Nur77 (a gift from Jeffery Milbrandt, Washington University, St. Louis, MO), rabbit polyclonal anti-V5 (Abcam), mouse monoclonal anti-myc (Cell Signaling), rabbit polyclonal anti-Nur77 M-210 (Santa Cruz Biotechnology), or rabbit polyclonal anti-Trx1 (Cell Signaling, Danvers, MA) . Secondary antibodies included donkey anti-rabbit 1:3000 and sheep anti-mouse 1:3000 (Amersham Biosciences). Membranes were processed using Pierce Super Signal West Pico chemiluminescent solution (Pierce).

2.1.4 Dual-Luciferase Assay

500,000 PC12 cells/well were seeded onto collagen-coated 6 well plates. Cells were transfected the following day with 1ug/well of the NBRE luciferase reporter and 0.1ug/well of the pRL-TK renilla construct using Lipofectamine according to manufacturer instructions (Invitrogen). 2 days after transfection, cells were treated with 50mM potassium chloride (KCl), 20mM caffeine, 100 μM/200 μM hydrogen peroxide (H_2O_2), 10 μM antimycin A, 2.5 mM pyruvate/2.5 mM malate (P/M), 5 mM succinate and/or 2 mM dithiothreitol (DTT) for 8 hours and luciferase activity was assessed using the dual-luciferase kit (Promega).

2.1.5 Redox Western Blotting for Trx1 and ΔAF-1-NGFIB-myc

The redox state of Trx1 and ΔAF-1-NGFIB-myc was determined by analysis of cell extracts pre-incubated with AMS (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid) as previously described (Hirasaka, Lago et al.). Briefly, cells were either untreated or treated with 5mM DTT or 500 μM-5mM hydrogen peroxide and precipitated with 10% ice-cold trichloroacetic acid for 30 min at 4°C. Samples were centrifuged at 12,000g for 30 min, resuspended in 100% acetone and incubated at 4°C for 30 min. Following centrifugation at 12,000g for 10 min, the acetone was removed and protein pellets were dissolved in 20mM Tris/HCl, pH 8.0, containing 15 mM AMS and incubated at room temperature (25°C) for 3 hours. Trx1 and ΔAF-1-NGFIB-myc redox forms were separated by SDS-PAGE using a non-reducing loading buffer (300mM Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 0.05% bromophenol blue).

2.1.6 Site-Directed Mutagenesis

An active site double cysteine to serine mutant thioredoxin1 ($C_{32}S$, $C_{35}S$) was generated using the QuickChange Site Directed Mutagenesis kit (Stratagene) using the following primers, FP: CAGCCACGTGGAGTGGGCCTAGCAAAATGATCAAGCC, RP: GGCTTGATCATTTTGCTAGGCCCACTCCACGTGGCTG.

2.1.7 Co-Immunoprecipitation

Co-immunoprecipitation (IP) experiments were performed in Hek293T cells transfected with combinations of NGFI-B-myc, Trx1-V5, or the active site mutant Trx1-V5 using calcium phosphate. Cells were lysed with RIPA buffer containing protease and phosphatase inhibitors (Roche). Lysates using 1-2mg of protein/sample were adjusted to

500ul with lysis buffer and incubated with 2ug anti-V5 antibody (Abcam) or rabbit IgG (for controls) overnight at 4°C. Thirty microliters of protein G sepharose beads (Amersham Biosciences) were added to each lysate and incubated at 4°C for an additional 16 hours. Samples were washed six times in lysis buffer after which samples were prepared for SDS-PAGE as described above.

2.1.8 Endogenous Co-Immunoprecipitation

PC12 cells were seeded at 5 x 10^6 cells/plate and were either left untreated or treated with 20mM caffeine for 3 hours to induce endogenous NGFI-B. IP samples were then processed using a lysis buffer containing 50mM Tris-HCl, 150mM NaCl, 5mM EDTA, 1% Triton-X, and protease inhibitors. IP samples (500ug protein/sample) were then incubated with 2ug of anti-Nur77 m-210 antibody (Santa Cruz Biotechnology) or IgG (for controls) at 4°C for 4 hours with rotation. IP sampled were then processed as described above and endogenous Trx1 was immunoblotted using anti-Trx1 antibody (Cell Signaling).

2.1.9 Statistics

Statistical evaluation of the data was performed using ANOVA followed by a Dunnett's post hoc test with a significance set *a priori* as $p<0.05$. Data represent averages +/- standard error of the mean (SEM) from at least 3 independent experiments.

2.2 METHODS AND MATERIALS FOR CHAPTER 4

2.2.1 Plasmid construction

The full-length coding sequence of mouse NGFI-B was sub-cloned into the pcDNA6/myc-His vector (Invitrogen) in using EcoRI and XhoI. Full-length mouse NGFI-B was also cloned into the FG9 lentiviral vector (kindly provided by Dr. Casey W. Wright, UT-Austin, TX, USA). The myc-tagged Bax vector was provided by Dr. Shawn Bratton, UT-Austin, TX, USA).

2.2.2 Cell Culture and Transfection assays

Rat L6 skeletal muscle cells and HeLa cells were cultured in DMEM growth medium containing 10% fetal bovine serum (Gemini) and 1% penicillin/streptomycin (Gibco). All transient transfection assays were carried out according to manufacturer instructions using TransIT®-LT1 transfection reagent (Mirius).

2.2.3 Lenti-viral Infection

Hek293T cells were seeded in at approximately 5 million cells per dish and allowed to adhere overnight in DMEM + 10% FBS. The next morning, cells were transfected (via calcium chloride transfection) with 5µg each of the viral packaging constructs FG9-CMV, FG9-RRE, FG9-REV, and 5µg of either FG9-empty vector (FG9-EV) or FG9-NGFI-B per plate of HEK cells. Briefly, all four constructs for each transfection were diluted in 1.5 mL sterile, DEPC treated ddH2O with 250mM CaCl2 (final concentration). DNA/CaCl2 mixture was added to an equal volume of 2x HBS while mixing vigorously and then allowed to incubate at room temperature for 15 minutes. Old media on the Hek293T plates was replaced with 9mL of DMEM + 10%

FBS + 25μM chloroquinone. 1 mL of transfection mixture was added drop-wise to each plate and swirled to mix. Plates were incubated at 37°C for 7 hours, then checked for precipitate formation to confirm successful transfection, and media was changed to 7mL of DMEM +10% FBS. Cells were then cultured in media supplemented with hygromycin for 1 week, then harvested via trypsinization and transferred to 10cm dishes. Cells were allowed to grow for an additional 2.5 weeks, until single colonies could be selected and screened.

2.2.4 Assessment of MMP by fluorescent microscopy

L6 skeletal muscle cells, 50,000 cells/glass chamber well, were transfected with either pEGFP or GFP-NGFI-B using TransIT®-LT1 transfection reagent (Mirius). After 24, 48, and 72 hours cells were stained with 25 nM tetramethylrhodamine, methyl ester, perchlorate (TMRM; Molecular Probes) for 10 min in the dark at 37°C, washed three times with Hanks balanced salts solution (HBSS, Sigma) and imaged using fluorescent microscopy (Nikon Eclipse TI-S).

2.2.5 Assessment of MMP by flow cytometry

L6 muscle cell lines ($1x10^6$) stably expressing either empty FG9 vector or FG9-NGFI-B were incubated with tetramethylrhodamine, methyl ester, perchlorate (25 nM TMRM; Molecular Probes) for 10 min in the dark at 25°C. Cells exhibiting red ($\lambda_{ex}/\lambda_{em}$= 488/ 620 nm) were assayed by flow cytometry (Beckman-Coulter FC500).

2.2.8 Mitochondrial cytochrome C release

L6 myoblasts (2 x 10_6) were transfected with empty pcDNA6/myc-His vector (Invitrogen), myc-tagged NGFI-B, myc-tagged Bax or exposed to UVB irradiation (5 min on a UV illuminator), washed with PBS, and resuspended in 100 µL of digitonin lysis buffer (75 mM KCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 60 µg/mL of digitonin) for 10 min on ice. Cytosolic fractions were then collected by centrifugation (15,000 x *g*; 10 min) and immunoblotted with a mouse anti-cytochrome c antibody (clone 6H2.B4, BD Pharmingen).

2.2.9 Whole cell oxygen consumption

Oxygen consumption analyses were performed (N>3 times in triplicate) using a Clark-type electrode as described previously (Brand *et al*., 2005; Hoppel *et al*., 1979) with minor modification. Briefly, respiration of 4 x 10^6 L6 muscle cells stably expressing either empty FG9 or FG9-NGFI-B were assayed in 1 mL Hanks balanced salts solution (HBSS, Sigma) by the sequential addition of oligomycin (1µg/ml) and dinitrophenol (DNP, 250µM). Slopes were recorded and statistical analysis using a student t test was performed.

Chapter 3: Redox regulation of the transcription factor NGFI-B

3.1 Introduction

ROS generated at supraphysiologic levels can indiscriminately oxidize and damage lipids, DNA, and proteins. Alternatively, the ability of the cell to control redox homeostasis has supported the hypothesis that ROS are discretely controlled and therefore serve as second messengers. This balance allows the cell to respond to slight changes in the redox state for the purpose of altering gene expression and modifying signaling pathways, thereby navigating the cell's fate (Allen and Tresini 2000; Balaban, Nemoto et al. 2005; D'Autreaux and Toledano 2007; Genestra 2007). Oxidants are capable of directly and indirectly altering cell function, whether it be the oxidant burst in response to growth factors that deactivate phosphatases and allow for the propagation of kinase signaling (Lee, Kwon et al. 1998; Rhee, Bae et al. 2000; Kwon, Lee et al. 2004) or by directly altering transcription factors in response to oxidative stress to translocate into the nucleus and transcribe antioxidant genes (Allen and Tresini 2000). While a wide array of pathways have been well characterized within the cell further understanding of how these pathways are coordinated to one another and to the cells needs remains unclear.

Our research reveals one potential redox target is the transcription factor NR4A1 also known as NGFI-B, Nur77, and TR3. The NR4A family, like other steroid receptors contains an N-terminal transactivation domain, a zinc finger DNA-binding domain, and a C-terminal ligand-binding domain (Hsu, Zhou et al. 2004). However, three-dimensional studies of the structure of NGFI-B and its closest relatives, Nor1 and Nurr1, have

revealed the NR4A family contains an atypical ligand-binding domain rendering it unable to bind stereotypical ligands such as steroids, fatty acids, retinoic acids, as well as other lipophillic molecules (Maxwell and Muscat 2006) (Wang, Benoit et al. 2003). Hence, the NR4A family members have been considered orphan nuclear hormone receptors because no physiological ligand has been identified that regulates their functions. The majority of research suggests the activity of the NR4A family members is mainly controlled by the level of protein induction and posttranslational modifications (Wansa, Harris et al. 2003) (Pei, Castrillo et al. 2006). NGFI-B also belongs to a small group of transcription factors capable of translocating to both the nucleus where it regulates gene expression and to the mitochondria where it is involved in the induction of apoptosis (Li, Kolluri et al. 2000; Lin, Kolluri et al. 2004). Even more interesting is the fact several of the stimuli involved in NGFI-B induction and activation are also associated with increased levels of intracellular ROS (Sundaresan, Yu et al. 1995; Bae, Kang et al. 1997; Colavitti, Pani et al. 2002) (Slater, Nobel et al. 1995). A variety of disease states for which NGFI-B is heavily involved in also reveal an integral role for redox disruption, including cardiac ischemia, atherosclerosis, and carcinogenesis (Cheng, Volkers et al.; Zhao, Howatt et al.; Wu, Liu et al. 2002; Pei, Castrillo et al. 2005; Mullican, Zhang et al. 2007).

Here, we demonstrate that the NGFI-B-mediated transcriptional regulatory pathway plays a novel role as a signal transduction relay between cellular redox status and the regulation of gene expression. Notably, both exogenous oxidants and physiologically-relevant NGF-B-inducing stimuli could stimulate NGFI-B induction and activity in multiple cell types. Correspondingly, experiments also demonstrate NGFI-B-

dependent transcription is potently inhibited by thiol-reducing stimuli through a novel identified interaction between NGFI-B and the cellular thiol reducing enzyme thioredoxin 1 (Trx1).

3.2 RESULTS

3.2.1 Redox regulation of NGFI-B induction

NGFI-B is encoded by an immediate early gene (NR4A1) that is rapidly induced by a variety of physiologic and pharmacologic stimuli, including potassium chloride (KCl), platelet derived growth factor (PDGF), epidermal growth factor (EGF), nerve growth factor (NGF), and caffeine (Milbrandt 1988; Katagiri, Hirata et al. 1997; Lammi and Aarnisalo 2008; Martin, Boucher et al. 2009). To test the hypothesis that NGFI-B expression is redox-sensitive, we treated both rat pheochromocytoma (PC12) and vascular smooth muscle (VSMC) cells with a range of doses of hydrogen peroxide and quantified NGFI-B protein levels using immunoblotting. Low micromolar concentrations of exogenous hydrogen peroxide (100 μM and 200 μM H_2O_2) significantly induced NGFI-B in a dose and time-dependent manner in both PC12 and VSMC cultures (Figure 3.1A,B, and 3.2B). To define the physiological relevancy of oxidant-induced NGFI-B induction, cells were pretreated with the thiol reducing agent DTT and subsequently treated with the NGFI-B-inducing stimuli caffeine, KCl, EGF, or NGF. As shown in Fig. 3.2 A and B, DTT pre-treatment significantly attenuated the induction of NGFI-B in both PC12 cells and VSMC cells by EGF, NGF, and caffeine, but not by KCl (Figure 3.2A,B). Thus, the physiological induction of the NR4A1 immediate early gene exhibits complex regulation by both redox-sensitive and redox-independent mechanisms.

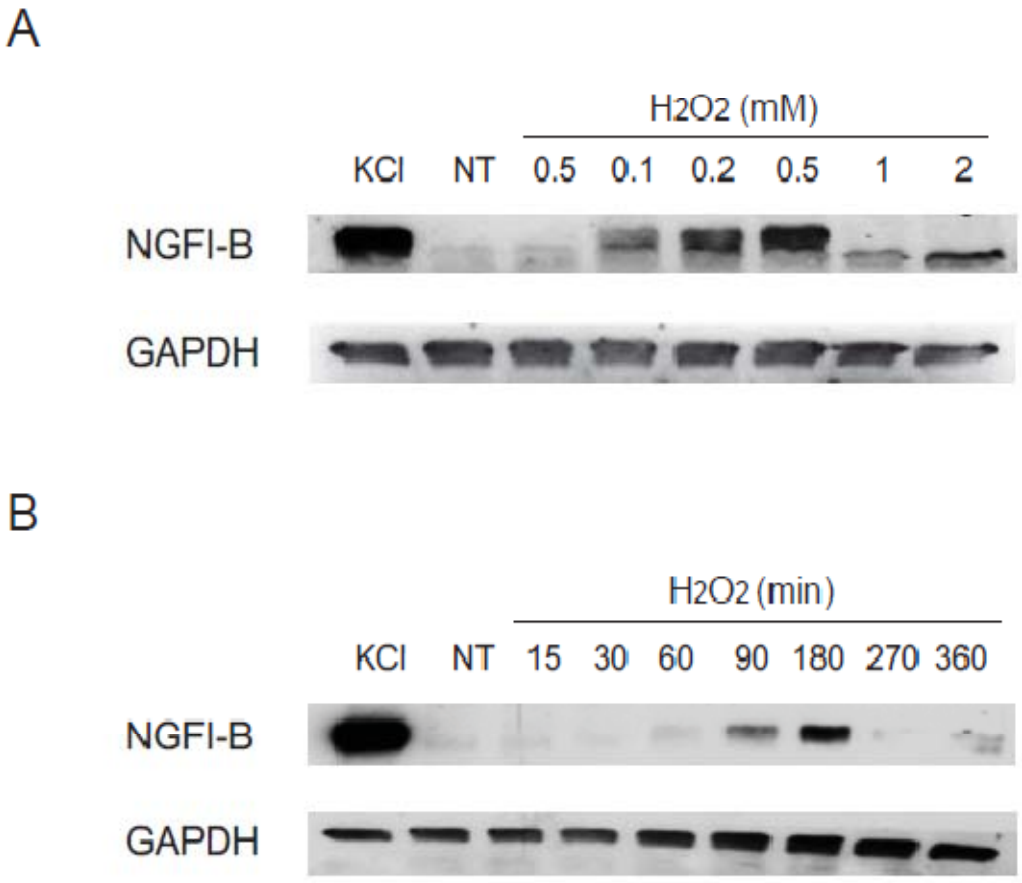


Figure 3.1 Regulation of NGFI-B induction

(A) Western blot of NGFI-B protein levels in rat pheochromocytoma (PC12) cells in response to no treatment (NT) or treatment of cells with the positive control KCl (50 mM), or a range of doses of hydrogen peroxide for 180 min.

(B) Time-course of NGFI-B protein induction in PC12 cells in response to 200 µM hydrogen peroxide. (A-B) For both western blots glyceraldehyde 3-phosphate dehydrogenase (GAPDH, bottom) protein levels were used to control for total protein on the membrane

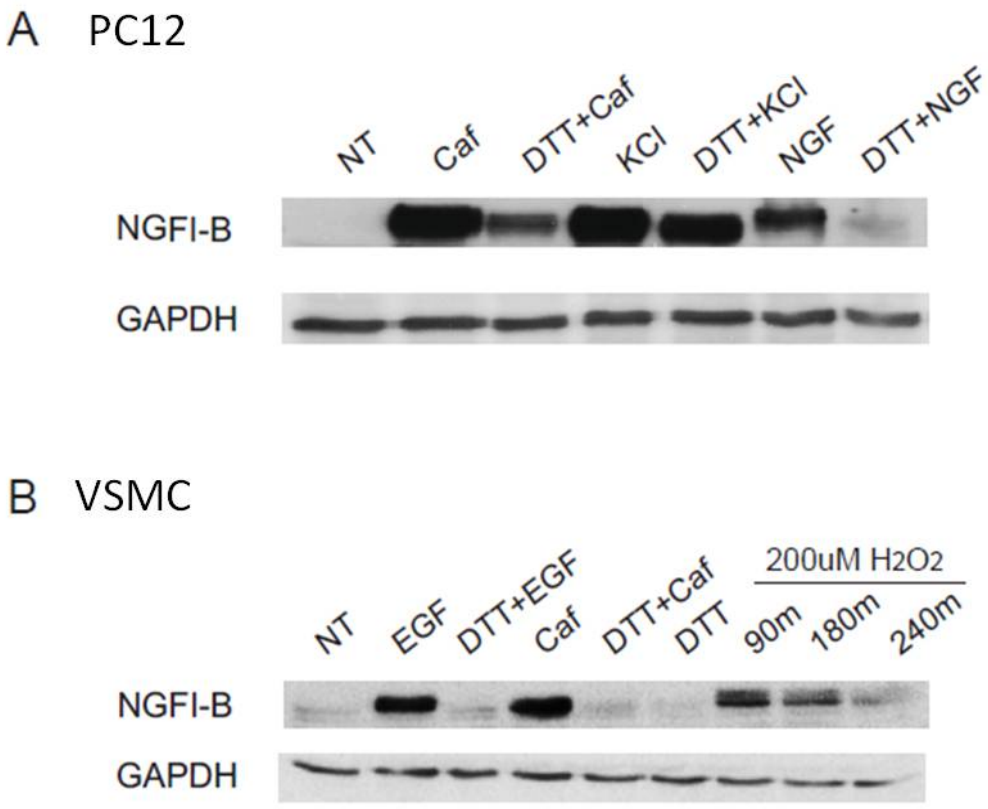


Figure 3.2 DTT inhibits NGFI-B induction by physiological inducers

(A) Effects of the thiol reducing agent, DTT (2 mM, 30 min.) on NGFI-B by induction in PC12 cells in response to caffeine, KCl, or NGF.

(B) Effects of DTT pretreatment (2 mM, 30 min.) on NGFI-B induction in response to hydrogen peroxide (200 µM, 90, 180 min.), caffeine (20 mM, 180 min) and EGF (100 ng/uL, 90 min) in VSMC cells. (A-B) For both western blots glyceraldehyde 3-phosphate dehydrogenase (GAPDH, bottom) protein levels were used to control for total protein on the membrane.

3.2.2 Redox regulation of NGFI-B-driven gene expression

Having established that NGFI-B expression is redox-sensitive, we next tested whether the transcriptional regulatory function of NGFI-B was also redox-controlled. NGFI-B binds to DNA as a monomer at the NGFI-B response element (NBRE) and as a homodimer or heterodimer with the other NR4A family members (Nurr1 and Nor1) to the Nur77 response element (NuRE). Canonically, these palindromic binding sequences consist of the octanucleotide AAAGGTCA motif for the NBRE, and the everted repeat AAAT(G/A)(C/T)CA motif for the NuRE. NGFI-B can also heterodimerize with the steroid receptor RXR and bind to the DR5 response element (Lam, Zhang et al.; Milbrandt 1988; Yoon and Lau 1993; Philips, Maira et al. 1997; Drouin, Maira et al. 1998; Wansa, Harris et al. 2002). As a functional assay to test the redox sensitivity of monomeric NGFI-B-dependent gene transcription, we used a previously described luciferase gene reporter construct (JA982/NBRE-luc) containing three contiguous NBRE sequences upstream of the luciferase gene (Katagiri, Hirata et al. 1997; Katagiri, Takeda et al. 2000). PC12 cells were co-transfected with the NBRE-luc construct along with the renilla luciferase internal control (pRL-tk) to normalize for changes in transfection efficiency. Both 100 μM and 200 μM hydrogen peroxide sharply induced NGFI-B-dependent luciferase expression (Figure 3.3A). Similarly, NBRE-luc expression was also induced by feeding cells the complex I substrates pyruvate/malate or the complex II substrate succinate in the presence of the complex III inhibitor antimycin, each of which drives complex III-dependent ROS production (Figure 3.3B) (Nemoto, Takeda et al. 2000). We next defined the impact of thiol reduction on NGFI-B-dependent transcription using the NBRE-luc / renilla-luc dual luciferase system to monitor gene transactivation in response to either heterologous NGFI-B-myc expression or endogenous NGFI-B induction. DTT treatment of PC12 cells significantly inhibited NGFI-B-myc-dependent

luciferase expression compared to untreated NGFI-B-myc transfectants (Figure 3.4A). Similarly, DTT pretreatment also significantly decreased KCl-induced NGFI-B-driven luciferase expression (Figure 3.4B). Since DTT had no effect on the capacity of KCl to induce NGFI-B protein (Fig. 3.2A), its inhibitory effect on endogenous NGFI-B-dependent transactivation of the luciferase reporter suggests that NGFI-B transcriptional functions may be generally redox sensitive. To rule out the possibility that DTT was causing a global non-specific effect on transcriptional machinery, we assessed the capacity of DTT to inhibit cyclic AMP response element-driven reporter activity using a CRE-luc cAMP reporter (Salvemini, Mazzon et al.). In PC12 cells expressing the CRE luciferase reporter and the renilla control, 20mM caffeine induced strong luciferase expression. However, thirty minute pre-treatments with either 1 or 2mM DTT failed to inhibit CREB-dependent gene expression, arguing that the effect of DTT on NGFI-B-dependent transcription is specific to NGFI-B (Figure 3.4C).

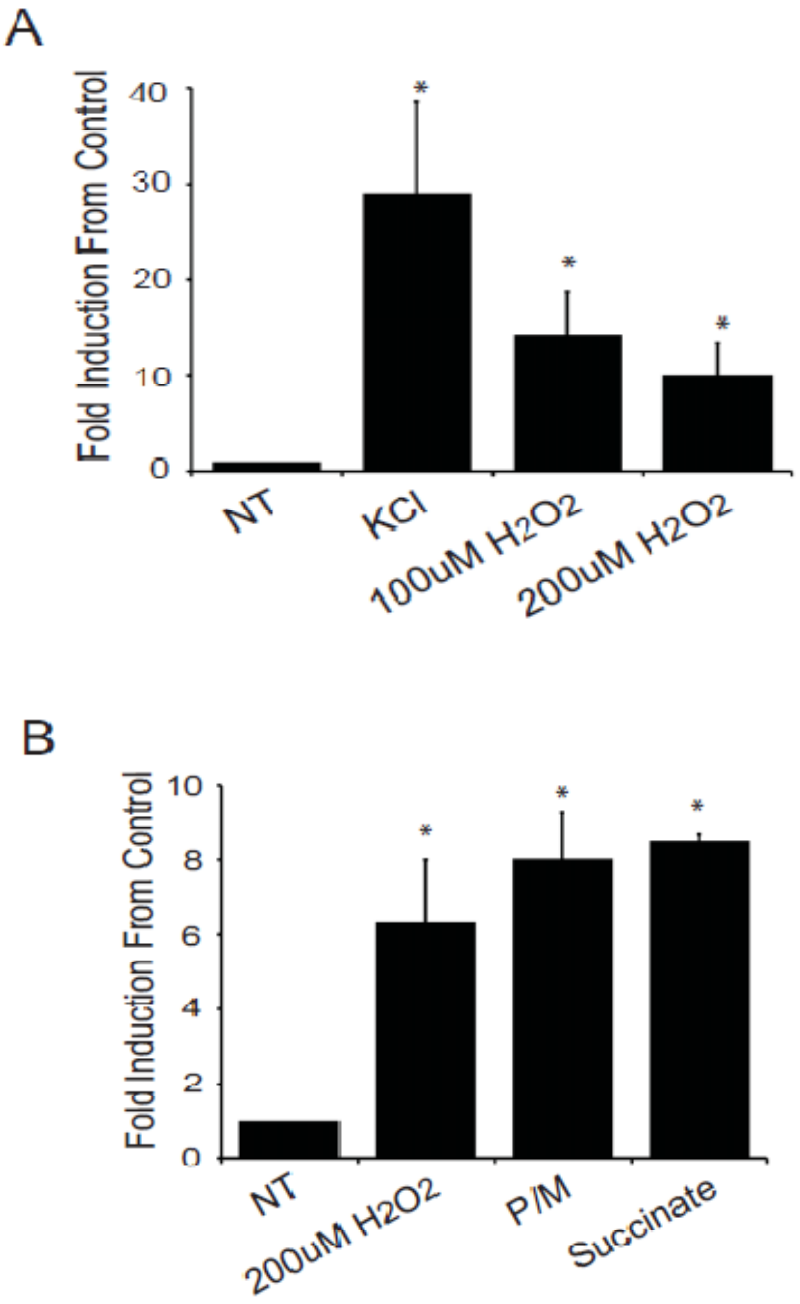
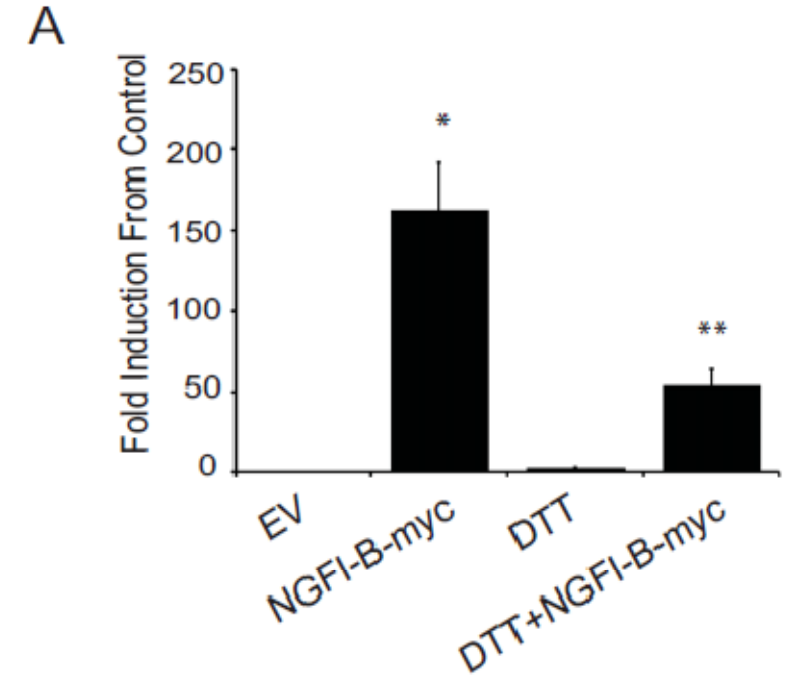
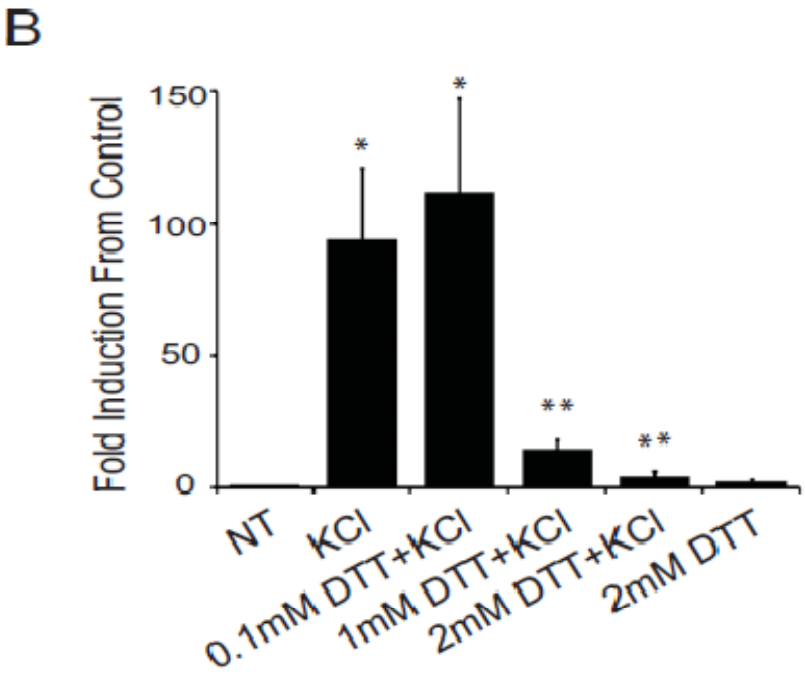


Figure 3.3 Redox regulation of NGFI-B-mediated transcription

(A) Dual luciferase assay (NBRE-luc reporter JA982) of NGFI-B dependent gene expression in PC12 cells alone (NT) or in response to treatments (8 hour) with KCl (50 mM, positive control) or hydrogen peroxide (100-200 μM).

(B) Dual luciferase assay (NBRE-luc reporter) of NGFI-B dependent gene expression in PC12 cells alone (NT) or in response to treatment (8 hour) with mitochondrial fuel substrates succinate (5 mM), pyruvate + malate (P/M, 2.5 mM), or hydrogen peroxide (200 μM). Results of luciferase activity in bar graphs are shown as means ± SEM (n=3, * = $p<0.05$).


A
Fold Induction From Control
250
200
150
100
50
0
*
**
EV
NGFI-B-myc
DTT
DTT+NGFI-B-myc


B
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150
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NT
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2mM DTT+KCl
2mM DTT

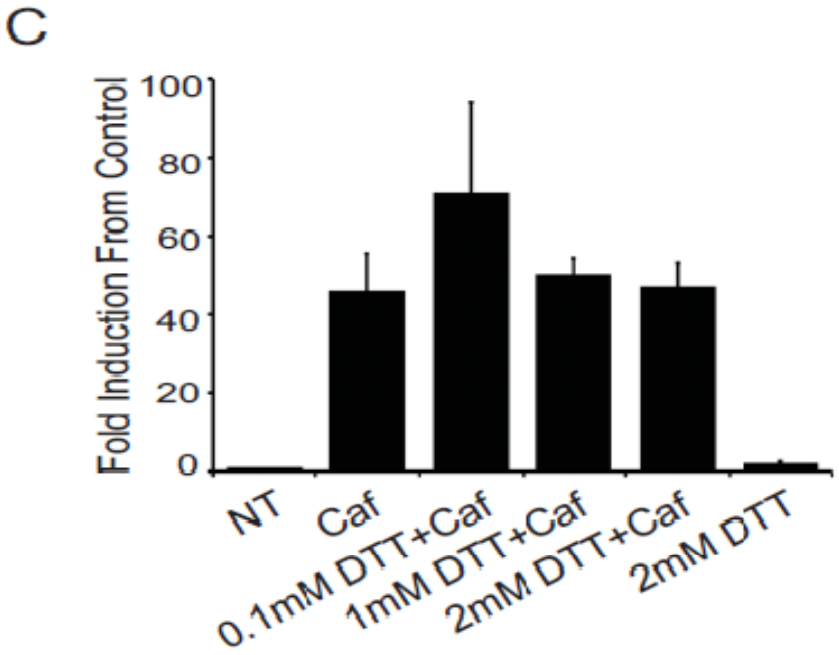


Figure 3.4 Effects of DTT on NGFI-B-dependent transcription

(A) Dual luciferase assay (NBRE-luc reporter) of the effects of DTT on NGFI-B-dependent gene expression in PC12 cells expressing either empty vector (EV) or full length NGFI-B-myc and treated with DTT (2 mM, 8 hrs.).

(B) Dual luciferase assay (NBRE-luc reporter) of the effects of DTT (2 mM, 30 min) on NGFI-B-dependent gene expression in PC12 cells alone (NT) or in response to 8 hour treatment with the indicated concentrations of KCl.

(C) Dual luciferase assay of the effects of DTT on caffeine-mediated, cyclic AMP response element reporter (CRE-luc reporter) activity in PC12 cells alone (NT) or in response to the indicated treatments with caffeine and DTT. Results of luciferase activity in bar graphs are shown as means ± SEM (n=3, * = $p<0.05$)

3.2.3 Hydrogen peroxide fails to block AMS alkylation of NGFI-B cysteines

To begin to understand the mechanisms underlying the redox sensitivity of NGFI-B function, a cysteine alkylation assay was performed using 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) that has been used to monitor cysteine oxidation in a variety of proteins, including Trx-2 (Hirasaka, Lago et al.). AMS binds to free, reduced cysteine thiols and increases the size of protein targets per alkylation event by approximately 500 Daltons. AMS binding is prevented if a reactive cysteine exists in any of various oxidized forms (disulfide bridges, hydroxylation, or glutathiolation, etc.). HEK-293T cells were transfected either with a mutant NGFI-B lacking the activation domain 1 (ΔAF-1-NGFI-B-myc) or Trx1-V5 (positive control) and were subsequently treated with 5mM DTT or various concentrations of hydrogen peroxide (ranging from 500 μM – 5 mM) prior to TCA precipitation and lysis in the presence or absence of AMS. Immunoblotting for Trx1-V5 revealed that AMS labeling occurred in the untreated (lane 2) and DTT-treated cells (lane 4), but not in hydrogen peroxide-treated cells (lane 3) (Figure 3.5A). In contrast, no hydrogen peroxide-dependent blockade of AMS labeling was observed with ΔAF-1-NGFI-B-myc (Figure 3.5B). Despite the presence of numerous cysteines, these data suggest that direct cysteine oxidation is not a mechanism of NGFI-B redox regulation. These data leave open the possibility of indirect redox regulation of NGFI-B through redox control of phosphorylation cascades and/or interaction with redox regulated proteins.

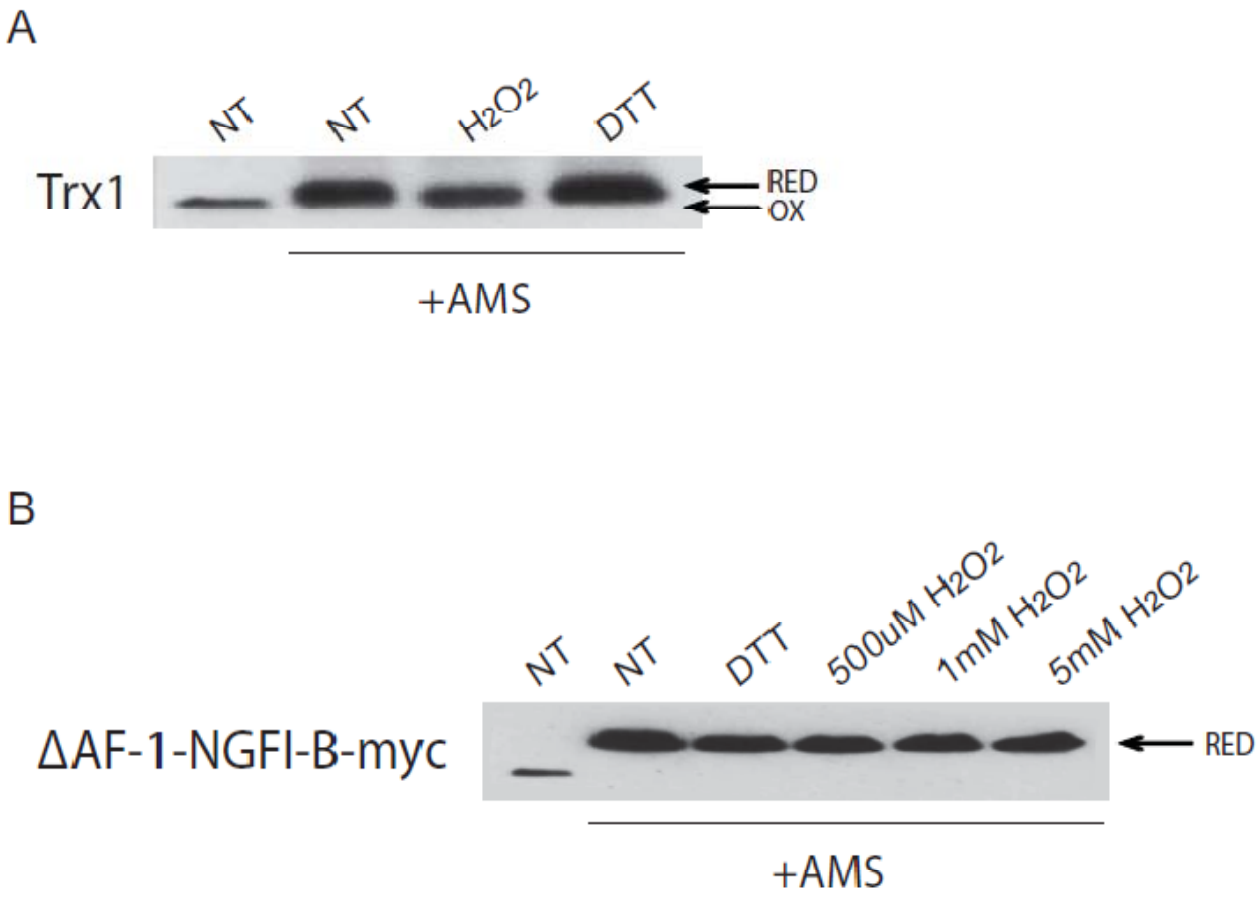


Figure 3.5 Redox western blotting of Trx1 and ΔAF-1-NGFI-B

Hek293T cells were transfected with Trx1-V5 (positive control) (A) or ΔAF-1-NGFI-B-myc (B) and were either untreated (NT) or treated with hydrogen peroxide (30 min) at the indicated doses or DTT (5 mM, 30 min) followed by lysate labeling with AMS as indicated. AMS bound to the upwardly shifted reduced (RED) but not peroxide-treated, oxidized (OX) forms of Trx1, but no oxidized non-AMS labeled species of ΔAF-1-NGFI-B-myc were detected.

3.2.4 Interaction of NGFI-B and Trx1

Given the effects of chemically-mediated thiol reduction on NGFI-B expression and activity, we reasoned that NGFI-B function may be regulated by thioredoxin-1 (Trx1), the most important and physiologically relevant cytoplasmic / nuclear thiol reducing enzyme system. Full-length NGFI-B-myc and Trx1-V5 were co-transfected (equimolar) in Hek293T cells and lysates were subsequently immunoprecipitated with anti-V5 antibody and immunoblotted for anti-myc. As shown in Figure 3.6A (lane 1), NGFI-B-myc was immunoprecipitated specifically by anti-V5 (Trx1-V5), but not by the negative control IgG (Fig. 3.6A, lane 2). Interestingly, 30 minute pre-incubation of Trx1-V5 and NGFI-B-myc expressing cells with hydrogen peroxide prior to cell lysis augmented the interactions compared to untreated cells (Fig. 3.6A, lane 3). In contrast, DTT failed to affect the Trx1-V5:NGFI-B interaction (Fig. 3.6A, lane 4). To rule out that the Trx1:NGFI-B complex formed in co-transfected cells was not a result of overexpression, we also performed immunoprecipitation experiments with the endogenous proteins. To induce NGFI-B protein, PC12 cells were stimulated with 20mM caffeine. Subsequently, lysates were immunoprecipitated with either an anti-NGFI-B (M-210) antibody (sc5569) or IgG. As shown in Figure 4B, anti-NGFI-B, but not IgG, immunoprecipitated endogenous Trx1, and this occurred only in cells where endogenous NGFI-B was induced (Fig. 3.6B, lane 3).

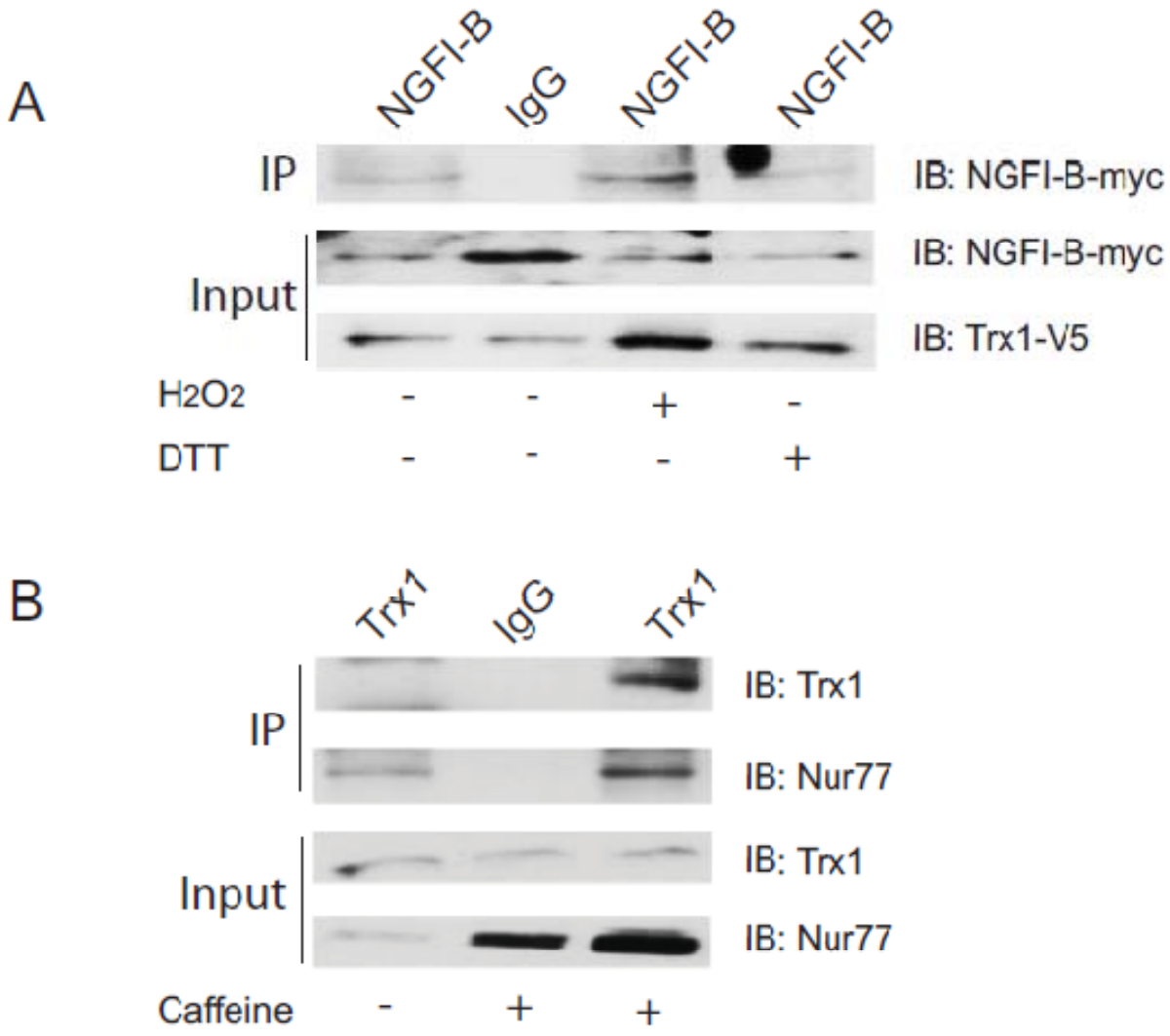


Figure 3.6 Complex formation between NGFI-B and Thioredoxin 1

(A) Co-immunoprecipitation assays in Hek293T cells co-transfected with NGFI-B-myc and Trx1-V5 and immunoprecipitated (IP) with either anti-Trx1-V5 or the negative control IgG (upper panel, lane 2) followed by immunoblotting (IB) as indicated (upper panel). Cells were either untreated (NT) or treated with hydrogen peroxide (1 mM, 30 min) or DTT (2 mM, 30 min) as indicated prior to

lysis immunoblotting (IB) for NGFI-B-myc. The lower two panels show input immunoblots for the heterologously expressed proteins.

(B) Co-immunoprecipitation assays for endogenous NGFI-B and Trx1 interactions in PC12 cells alone (NT) or treated with caffeine (20 mM, 180 min) to induce NGFI-B prior to lysis. Lysates were immunoprecipitated with either anti-NGFI-B (lanes 1, 3) or IgG (lane 2) followed by immunoblotting (IB) for Trx1 and NGFI-B (upper two panels). The lower panels show the levels of constitutive Trx1, and NGFI-B before and after induction with caffeine (Input).

3.2.5 Mutational analysis of the NGFI-B/Trx1 complex

A canonical view of Trx1 redox-regulatory action is that it binds proteins via its active site cysteines as a requisite step in thiol reduction. We performed mutagenesis studies to characterize the mechanisms regulating the NGFI-B:Trx1 complex. The NR4A family share 14 conserved cysteine residues spanning the DNA-binding (DBD) and ligand-binding domains (LBD), but share none in the activation domain. Co-immunoprecipitation assays with full length Trx1-V5 and a mutant NGFI-B lacking the activation domain (ΔAF-1-NGFI-B-myc) revealed that the NGFI-B activation domain was dispensable for Trx1 binding (Figure 3.7A). However, after site-directed mutagenesis, we found that the active site cysteines ($C_{32}S$, $C_{35}S$) were absolutely required for NGFI-B-Trx1 binding (Fig. 3.7B, lanes 1-4). These data are consistent with the notion that Trx1 binds NGFI-B in a cysteine-dependent manner.

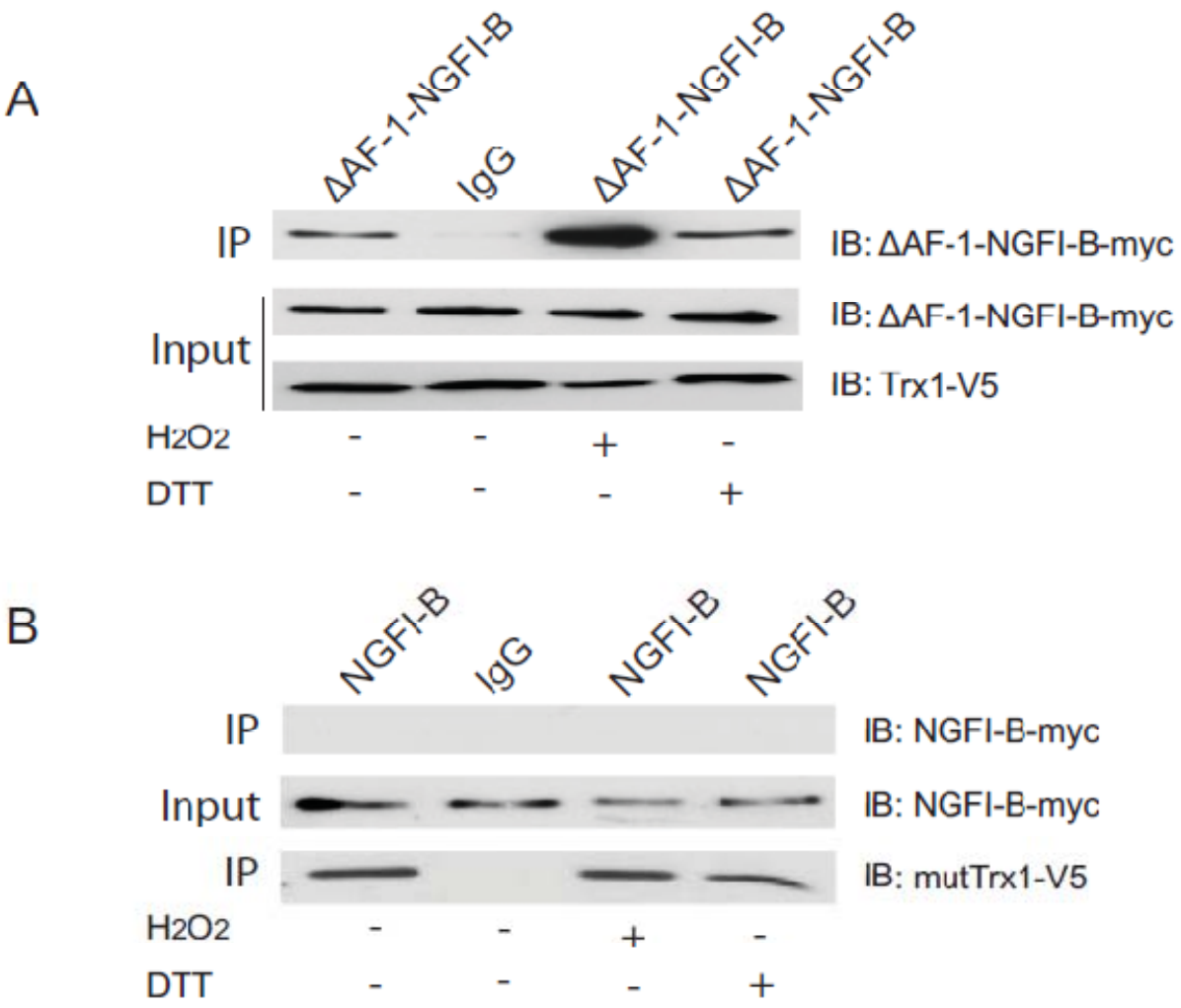


Figure 3.7 Mutational analysis of the NGFI-B/Trx1 complex

(A) Co-immunoprecipitation assays in Hek293T cells co-transfected with the ΔAF-1-NGFI-B-myc truncation mutant and Trx1-V5 and immunoprecipitated (IP) with either anti-Trx1-V5 or the negative control IgG (upper panel, lane 2) followed by immunoblotting (IB) as indicated (upper panel). Cells were either untreated (NT) or treated with hydrogen peroxide (1 mM, 30 min) or DTT (2 mM, 30 min) as indicated prior to lysis and immunoblotting (IB) for NGFI-B-myc.

(B) Co-immunoprecipitation assays in Hek293T cells co-transfected with the NGFI-B-myc and the active site cysteine mutant Trx1-($C_{32}S$,$C_{35}S$)-V5 were immunoprecipitated (IP) with either anti-Trx1-V5 or the negative control IgG (upper panel, lane 2) followed by immunoblotting (IB) as indicated (upper panel). Cells were either untreated (NT) or treated with hydrogen peroxide (1 mM, 30 min) or DTT (2 mM, 30 min) as indicated prior to lysis immunoblotting (IB) for NGFI-B-myc.

3.2.6 Trx1 inhibits NGFI-B-dependent transcription

Finally, we tested the effects of Trx1 on NGFI-B function. PC12 cells were co-transfected with the NBRE-luc and renilla-luc luciferase constructs in the presence or absence of NGFI-B-myc, Trx1-V5, and/or the active site mutant Trx1-V5, and luciferase transcriptional reporter assays were performed. As expected, NGFI-B-myc alone induced strong luciferase expression (Figure 3.8A). However, similar to the effects of DTT, Trx1-V5 sharply inhibited NGFI-B-dependent gene expression (Figure 3.8A). In contrast, the active site mutant Trx1 failed to inhibit NGFI-B-dependent reporter activity (Figure 3.8A). Next we tested the physiologic relevancy of these observations by defining the role of transfected Trx1 in the regulation of endogenous NGFI-B function in PC12 cells. Cells treated with caffeine alone exhibited a strong induction of NGFI-B reporter activity (Figure 3.8B). In contrast, caffeine-induced NGFI-B reporter activity was strongly decreased in the presence of wild type Trx1, but not mutant Trx1 (Figure 3.8B). Together, these results demonstrate that Trx1 is an inhibitor of NGFI-B-dependent, NBRE-regulated gene transcription.

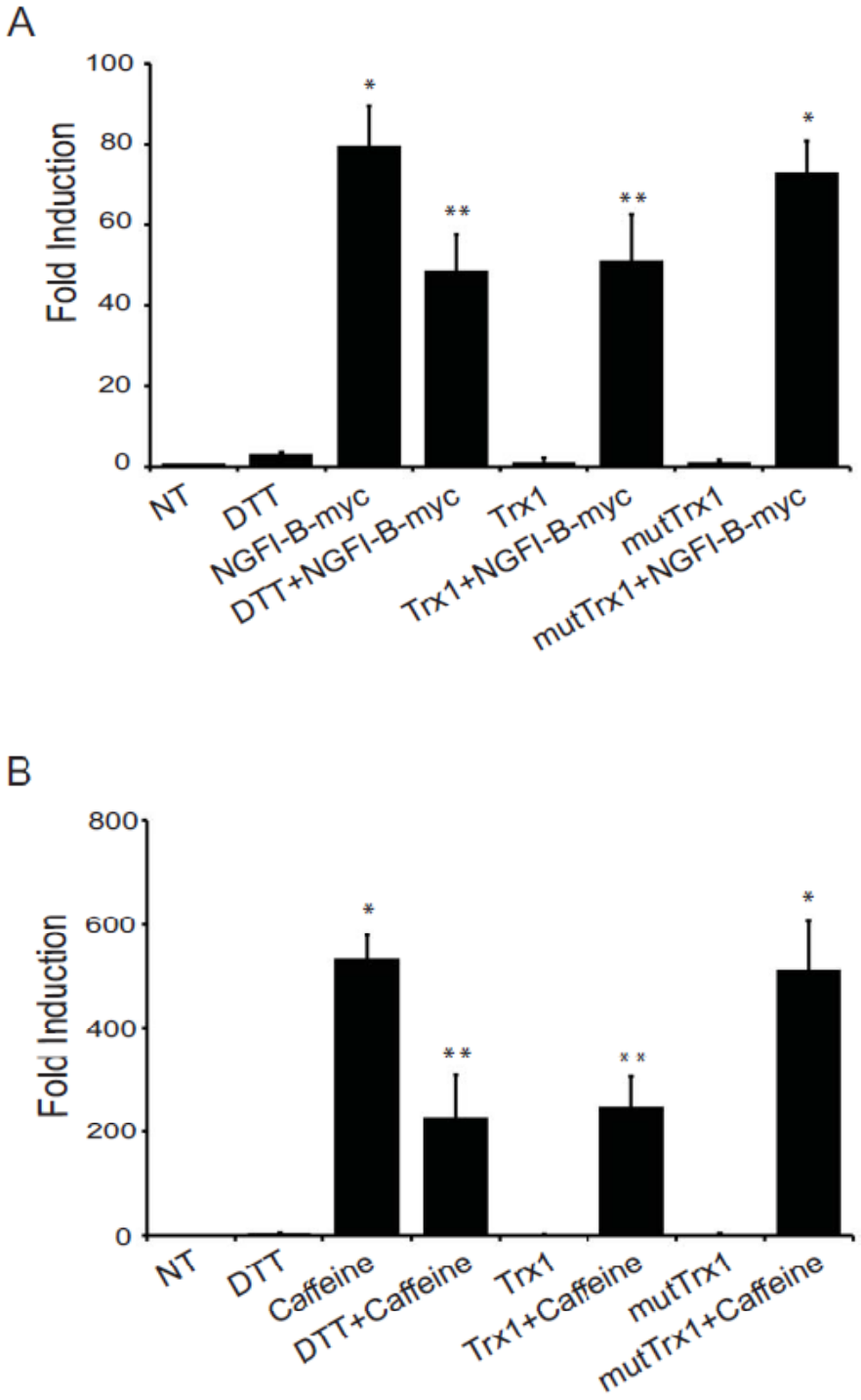


Figure 3.8 Effect of Trx1 on NGFI-B-dependent transcription

(A) Dual luciferase assay (NBRE-luc reporter JA982) of NGFI-B dependent gene expression in PC12 cells transfected with NGFI-B-myc along with either Trx1 or the active site mutant Trx1 ($C_{32}S$, $C_{35}S$)

(B) Dual luciferase assay (NBRE-luc reporter JA982) of NGFI-B dependent gene expression in PC12 cells transfected with either Trx1 or the active site mutant Trx1 ($C_{32}S$, $C_{35}S$) and treated with caffeine to induce endogenous NGFI-B-dependent transcription. Results of luciferase activities are shown as means ± SEM (n=3, * = $p<0.05$).

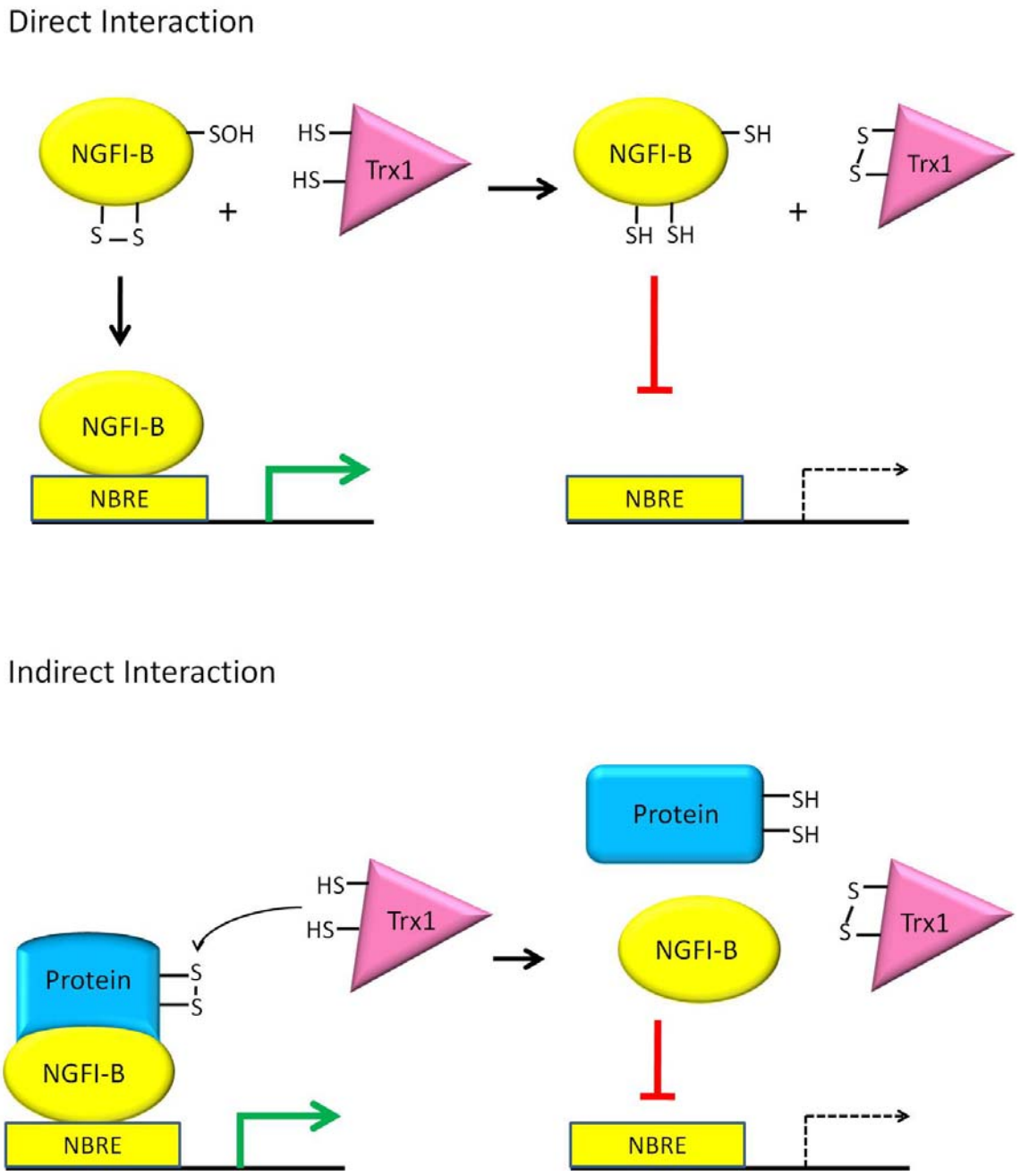


Illustration 7 Model of Trx1 regulated NGFI-B function

Like other transcription factors, Trx1, may regulate NGFI-B-dependent gene expression through a direct interaction with NGFI-B. Using co-immunoprecipitation assays we clearly show that NGFI-B and Trx1 interact and that the active site of Trx1 is necessary

for the interaction between Trx1 and NGFI-B to occur, however, we were unable to prove using the thiol alkylating assay that NGFI-B contains any reactive cysteines. While this does not rule out that a direct interaction could still be involved in the Trx1-dependent inhibition of NGFI-B transactivation it is necessary to uncover whether this interaction is direct through *in vitro* immunoprecipitation and whether NGFI-B can be directly modified by oxidants. Another possible mechanism by which Trx1 inhibits NGFI-B-dependent transcription is by binding and reducing another protein necessary for NGFI-B DNA binding and transcription. The oxidized form of this unknown protein readily binds and promotes NGFI-B-dependent gene expression but it is possible that once reduced by Trx1 or addition of the chemical thiol reducing agent, DTT that a conformation change prevents the binding of this protein and NGFI-B, thus inhibiting gene expression by NGFI-B.

3.3 DISCUSSION

Apart from its tissue-specific functions in cell growth, differentiation, and death, NGFI-B and its closely related NR4A paralogous nuclear receptors have attracted considerable recent interest for their potential roles in metabolic signaling and disease (Pearen and Muscat). The mechanisms coupling NGFI-B function to changes in metabolism are not well defined. One potential route through which cellular and mitochondrial energy metabolism may signal in cells is through alterations in cellular redox balance by, for example, modulation of mitochondrial oxidant generation and shifts in the NAD+/NADH redox couple (McLaughlin, Strain-Damerell et al.). Here, we demonstrate that NGFI-B signaling is strongly sensitive to both oxidative and reductive changes in cellular redox balance, and regulated, at least in part, through the formation of a complex with the thiol reductase enzyme Trx1. These studies indicate that the redox regulation of NGFI-B signaling occurs in a biologically redundant fashion to control both NR4A1 immediate early gene expression, and NGFI-B protein induction.

NGFI-B was first identified roughly two decades ago by J. Milbrandt as a nerve growth factor-inducible homolog of the glucocorticoid (GC) receptor in PC12 cells (Milbrandt 1988; Mangelsdorf, Thummel et al. 1995). Like other nuclear receptors,

NGFI-B and its close NR4A family paralogs Nurr1 and Nor-1 contain a ligand independent AF-1 transactivation domain and a DNA binding domain with two contiguous zinc finger DNA binding motifs, along with a ligand-binding domain containing another ligand-independent AF-2 transactivation domain ((Paulsen, Weaver et al. 1992), Illustration 3). Uniquely among nuclear receptors, NGFI-B and its closely related paralogs are classified as orphan nuclear receptors because no activating endogenous ligand has yet been identified for any member of the NR4A family. Moreover, the ligand-binding domain of NGFI-B has been confirmed by X-ray crystallography to lack the typical ligand docking pocket of other nuclear receptors, and instead contains exposed sterically bulky amino acid residues that exist in a closed confirmation (Wang, Benoit et al. 2003). Together, these observations have led investigators to conclude that NR4A1 gene induction, post-translational modification, and NGFI-B localization are the major modes of NGFI-B regulation. NGFI-B is an established substrate for multiple protein kinases and is post-translationally modified by complex patterns of phosphorylation on several amino acid residues in a stimulus-dependent manner resulting in the regulation of NGFI-B localization within the cell (Fahrner, Carroll et al. 1990; Katagiri, Hirata et al. 1997; Katagiri, Takeda et al. 2000; Masuyama, Oishi et al. 2001; Jacobs, Boldingh et al. 2004; Han, Cao et al. 2006). Additionally, NGFI-B is among only a few transcription factors identified, including p53 and DJ-1 / Park7 (Hao, Giasson et al.; Springer and Kahle; Mihara, Erster et al. 2003; Moll, Marchenko et al. 2006), that can be targeted to and regulate mitochondrial function (Li, Kolluri et al. 2000; Lin, Kolluri et al. 2004). Whereas our data strongly support the notion that NGFI-B function is redox-sensitive, neither exogenous micromolar peroxide nor reduction with DTT or Trx1 influenced the average sub-cellular nuclear to cytoplasmic distribution of GFP-tagged NGFI-B in PC12 cells. Future work will aim to

understand the sub-cellular distribution and spatiotemporal regulation of the NGFI-B / Trx1 redox-sensitive complex.

To our knowledge, this is the first work that demonstrates the capacity for NGFI-B and Trx1 to form a complex. However, other members of the steroid / thyroid nuclear receptor superfamily have been shown to be redox regulated. Recently it was reported that the GC receptor is redox-regulated by Trx1 (Makino, Yoshikawa et al. 1999). Unlike our results showing that DTT- and Trx1-dependent reduction of NGFI-B blocked its function, the transcriptional function of the GC receptor was inhibited by oxidative stress, and was restored in response to complex formation with Trx1 (Makino, Yoshikawa et al. 1999). This divergent redox regulation of GC and NGFI-B receptors is consistent with their functional antagonism for the control of transcription in general (Philips, Maira et al. 1997). Similarly, Trx1 has been shown to interact with and activate nuclear estrogen receptor alpha-dependent transcription (Hayashi, Hajiro-Nakanishi et al. 1997). Trx1 has also been demonstrated to activate transcription factors not belonging to the nuclear receptor family. For example, Trx1 was found to increase p53-dependent transcription (Jayaraman, Murthy et al. 1997), to stimulate AP-1-driven transcription and DNA binding by forming a complex with Ref-1 (Hirota, Matsui et al. 1997), and to increase NF-κB-dependent transcription via a complex with the p50 subunit of NF-κB (Qin, Clore et al. 1995). Our results showing that Trx1 and chemical reduction inhibited NGFI-B function (and induction) reveal a unique, inhibitory mechanism for Trx1 in transcriptional regulation.

The work presented here reveals that NGFI-B function is sensitive to redox regulation upstream of NGFI-B itself (e.g. NGFI-B expression). Trx1 contains a pair of vicinal cysteines in its active site (motif CVNVGC) that are required for its thiol reductase activity and typically for binding to substrates (Holmgren 1995; Weichsel,

Gasdaska et al. 1996). We found using immunoprecipitation experiments in cells that indeed Trx1 interacted with NGFI-B in an active site cysteine-dependent manner. However, results showing that hydrogen peroxide failed to block NGFI-B labeling with the thiol alkylating reagent AMS suggest that cysteine reduction might not be the mechanism through which Trx1 operates. Since Trx1 has also been shown to regulate protein nitrosylation (Wu, Parrott et al.), more work is needed to define the possible direct, biochemical mechanisms of Trx1-mediated NGFI-B regulation.

Finally, these results provide a new biochemical framework to begin to address potential mechanisms linking NGFI-B and cellular redox changes to similarly pleiotropic physiological and pathological processes. As reviewed by Maxwell *et. al.*, (Nuclear Receptor Signaling, 2005), NGFI-B responds to diverse stimuli that are also associated with increased production of cellular ROS, including prostaglandins, phorbol esters, fatty acids, cell stress, mechanical agitation, mitogenic growth factors, and inflammatory cytokines. Indeed, our observations that hydrogen peroxide alone could induce, and that DTT could inhibit, the induction of NGFI-B protein expression by caffeine, epidermal growth factor, and nerve growth factor confirms that the NR4A1 gene is likely to be broadly redox regulated. Though its physiologic functions are far from fully established, NGFI-B is involved in dopaminergic neuronal development, steroidogenesis, T-cell selection (apoptosis), and adipose development. Arguably, the most important disease association for NGFI-B is atherosclerosis, which is strongly linked to oxidative stress and vascular remodeling involving circulating macrophages, and vessel wall endothelial and smooth muscle cells (Zafari, Ushio-Fukai et al. 1998). In the vessel wall, NGFI-B activation is associated with pathologic vascular remodeling in response to tumor necrosis factor-1 alpha (TNF-α), in part by inducing the plasminogen activator-1 activator (PAI-1) gene, ostensibly through an NGFI-B response element in the 5’

upstream promoter region (Gruber, Hufnagl et al. 2003). Similarly, TNF-α is well known to increase endothelial ROS production; and increased vascular oxidative stress and PAI-1 levels each is associated with vascular disease risk (Li and Fukagawa). Our results showing that NGFI-B is induced in vascular smooth muscle cells are consistent with other reports that NGFI-B induction occurs in response to smooth muscle cell proliferative stimuli. Thus, reductive inhibition of NGFI-B in the vasculature may comprise a new therapeutic avenue for the prevention of neointimal formation, and plaque development during atherogenesis.

Together, these studies reveal an unappreciated role for NGFI-B as a mediator of a redox-sensitive pathway of gene regulation. Moreover, the studies add NGFI-B to an expanding portfolio of potential substrates for Trx1, and shed light on a novel, inhibitory function of Trx1 in the regulation of gene expression. This work lays the foundation for future work aimed at understanding in more detail the direct and indirect biochemical mechanisms underlying the redox regulation of NGFI-B.

Chapter 4: NGFI-B regulates mitochondrial membrane potential (MMP) in L6 skeletal muscle cells

4.1 INTRODUCTION

The involvement of nerve growth factor inducible protein B (NGFI-B) in the induction of cell death in a variety of cell lines is well established (Li, Kolluri et al. 2000; Holmes, Soprano et al. 2003; Kolluri, Bruey-Sedano et al. 2003; Holmes, Soprano et al. 2004; Lin, Kolluri et al. 2004; Lee, Ma et al. 2005; Maddika, Mendoza et al. 2006). It is now widely believed that NGFI-B induces apoptosis by localizing to the mitochondria through an interaction with Bcl-2 (Lin, Kolluri et al. 2004). While various studies have demonstrated mitochondrial translocation of NGFI-B followed by the release of cytochrome c, and the induction of apoptosis, full understanding of the mechanism underlying NGFI-B-dependent programmed cell death is still undetermined. An interaction between NGFI-B and Bcl-2 causes a conformational change in Bcl-2 that exposes its BH3 domain, but what events follow remain unclear. One could speculate that the removal of Bcl-2 from inhibitory role of other pro-apoptotic Bcl-2 family members such a Bax, Bid, and Bak could allow for Bax oligomerization and permeabilization of the outer mitochondrial membrane, resulting in cytochrome c release (Kluck, Esposti et al. 1999). However, researchers have not been able to demonstrate the intervening events that occur between the interaction between NGFI-B and Bcl-2, the subsequent conformational change, and the eventual release of cytochrome c. Therefore, the manner in which this interaction causes the release of pro-apoptotic factors from the mitochondria continues to be purely conjecture. The difficulty in uncovering answers to many of these questions is lies in the rapidity of apoptosis in general. Once caspases are activated, distinction between cellular alterations that lead to apoptosis and those that simply result from caspase induction become complicated.

Correspondingly, studies have revealed that the irreversible loss of the MMP often precedes programmed cell death, but even this event is not observed in an all or none fashion (Cossarizza, Kalashnikova et al. 1994; Hishita, Tada-Oikawa et al. 2001; Karpinich, Tafani et al. 2002) leading to contradictory views concerning whether the MMP is actively involved in apoptosis or simply one of the many indicators that programmed cell death has been initiated.

Temporary, reversible, loss of MMP appears to be involved in the regulation of intracellular calcium and could also be a means of regulating redox signaling within the cell. Maintenance of the MMP at -180 to -200 mV in respiring mitochondria drives the influx of calcium ions (Ca^{2+}) into the mitochondria (Rizzuto, Bernardi et al. 2000). This can create a sink for calcium and alter the availability of intracellular calcium for signaling purposes (Herrington, Park et al. 1996; Hoth, Fanger et al. 1997; Billups and Forsythe 2002). An influx of too much calcium into the mitochondria is thought to induce apoptosis and lead to the full opening of the MPTP and irreversible loss of the MMP (Bossy-Wetzel, Newmeyer et al. 1998). Lowered MMP also allows electrons to pass more efficiently through the ETC, thereby limiting the amount that slips to molecular oxygen and forming superoxide (Korshunov, Skulachev et al. 1997; Liu 1999). This can affect the redox poise within the cell and have an impact on redox signaling pathways. The involvement of mitochondria in redox signaling has only been established in the last five years, but the redox state of the cell has been well established to have an impact on the induction of apoptosis. Studies have not only correlated a rise in ROS to the induction of apoptosis, but directly linked increased ROS to the opening of the MPTP through oxidation of critical thiols on the matrix side of the adenine nucleotide transporter (ANT) (McStay, Clarke et al. 2002). Researchers have also discovered that Bax requires a pro-oxidant environment in order to form the disulfide bridges necessary

for homo-oligomerization (Brustovetsky, Li et al.). Previously we described evidence that NGFI-B induction and function are redox sensitive. Along with the pro-apoptotic function of NGFI-B, this provides support for the involvement of redox regulation in NGFI-B-dependent cell death.

Using the MMP-sensitive dye TMRM, we found that enforced NGFI-B expression in L6 skeletal myoblasts led to a significant loss of MMP that peaked 48hr after transfection. Similarly, stable expression of NGFI-B in L6 muscle cells resulted in prolonged loss of the MMP without any signs of cell death. The NGFI-B-induced decrease in MMP was specific to L6 myoblasts and was demonstrated using the MMP indicator tetramethylrhodamine methyl ester (TMRM). In the absence of a pro-apoptotic stimulus, NGFI-B was evenly distributed between the cytoplasm and nucleus in cells lacking MMP. NGFI-B expressing cells which lacked MMP were morphologically normal in comparison to their non-NGFI-B transfected neighbors displaying MMPs. Moreover, NGFI-B transfected cells had no increase in mitochondrial cytochrome C release 48hrs post-transfection. Transfection of L6 skeletal muscle cells with a transactivation domain mutant NGFI-B (ΔAF1-NGFI-B-myc) that is incapable of regulating gene transcription also led to a loss of MMP. Interestingly, treatment of NGFI-B transfected cells with the calcium ionophore, ionomycin, 24 hours after transfection and prior to the loss of MMP led to a rapid loss of MMP within 3 hours, specifically in NGFI-B-transfected L6 cells. Furthermore, oxygen consumption experiments on stable NGFI-B and empty vector (EV) L6 muscle cell lines revealed a significant increase in oxygen consumption nu NGFI-B L6 muscle cells only during State 3 respiration, and no difference demonstrated during State 4 respiration. Combined, these preliminary data suggest that NGFI-B-dependent loss of MMP in muscle cells may

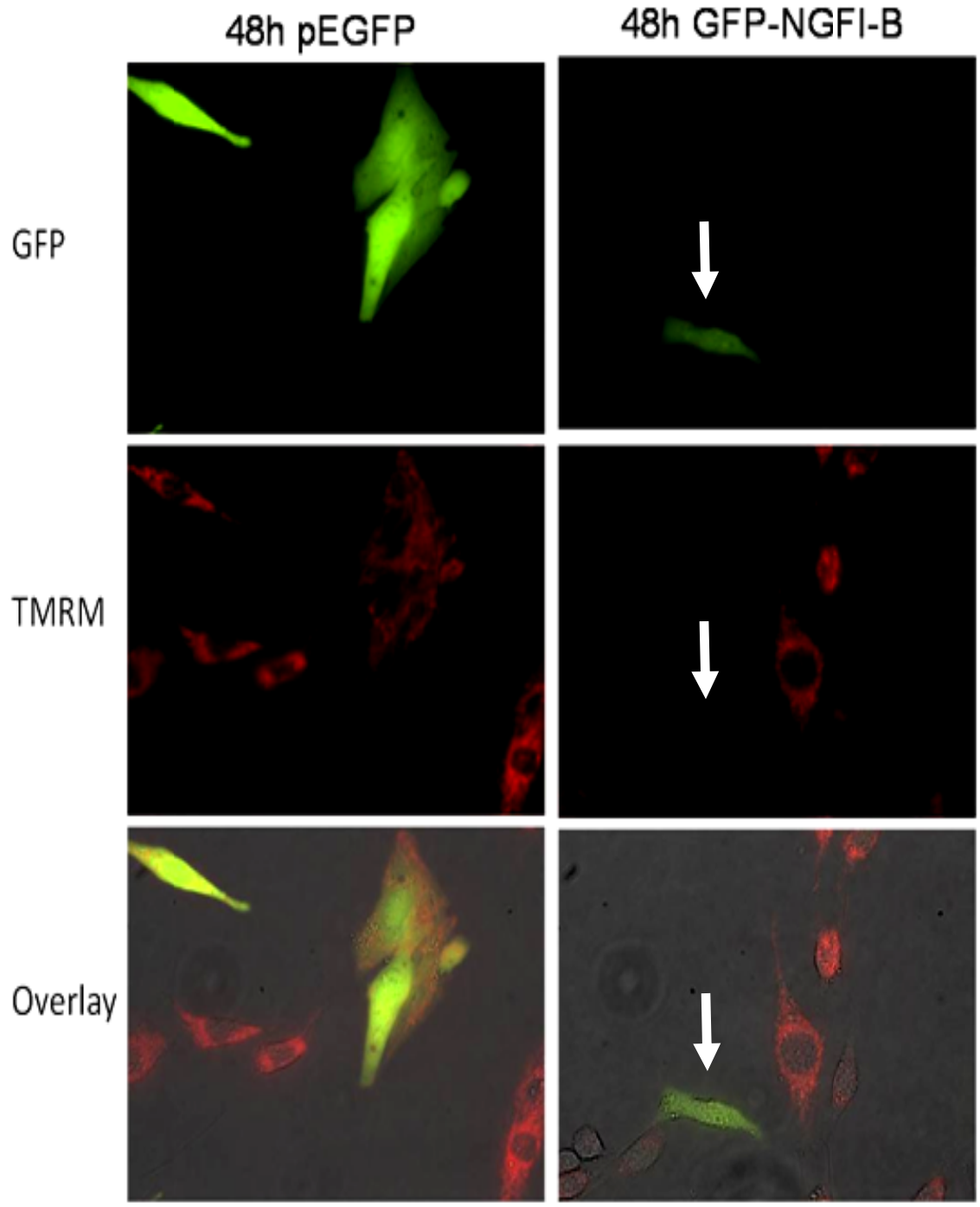
involve mitochondrial calcium handling and provide a possible mechanism through which NGFI-B regulates mitochondrial function.

4.2 RESULTS

4.2.1 Overexpression of NGFI-B lowers mitochondrial membrane potential

Muscle cells become resistant to cell death upon differentiation (Wang and Walsh 1996; Kamradt, Chen et al. 2002). We hypothesized that this could provide an opportunity to study NGFI-B-depedent regulation of mitochondrial function. L6 skeletal myoblasts, transfected with empty pEGFP vector or GFP-NGFI-B, were observed using fluorescent microscopy. GFP-NGFI-B exhibits normal subcellular localization despite the size of the GFP tag (Katagiri, Takeda et al. 2000). Non-treated (NT) GFP-NGFI-B transfected myoblasts demonstrated even distribution of GFP-NGFI-B throughout the cell while the pEGFP expressing cells expressed robustly within the cytosol (Figure 4.1A). The transfected cells were then stained with 25 nM TMRM and again observed by fluorescent microscopy. At 48 hours post-transfection cells expressing GFP-NGFI-B appeared to have little to no TMRM fluorescence suggesting a loss or lowering of the MMP (Figure 4.1A). These cells, however, remained morphologically similar to neighboring non-GFP-NGFI-B cells as well as those transfected with empty pEGFP displaying TMRM fluorescence (Figure 4.1A). Quantification of 100 cells from each treatment group confirmed that NGFI-B-dependent loss of MMP was indeed significant compared to cells transfected with empty pEGFP vector (Figure 4.1B).

A



B

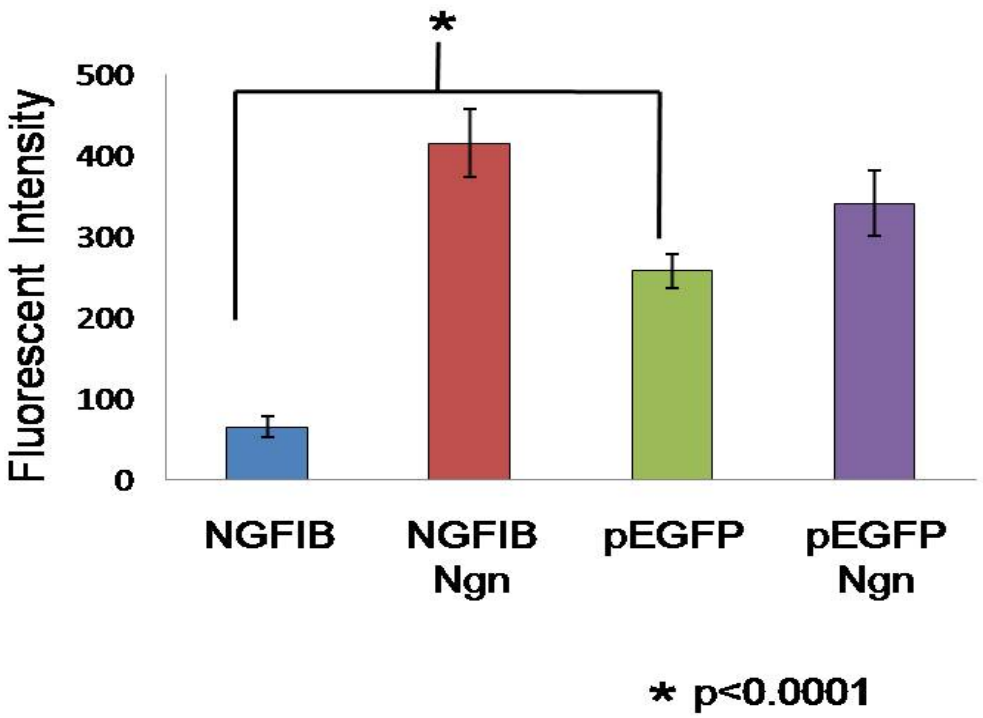


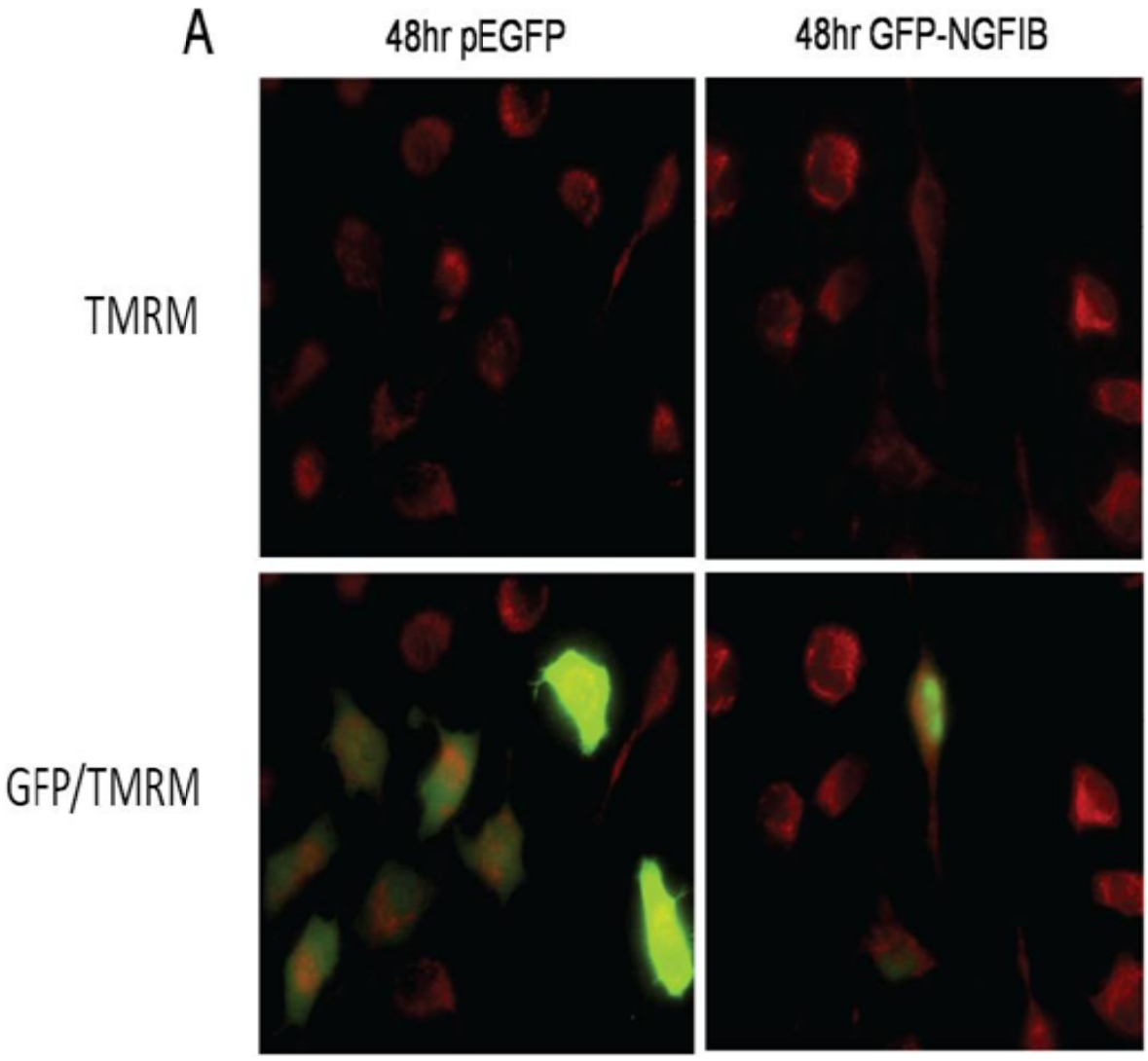
Figure 4.1 Effect of NGFI-B on MMP in L6 myoblasts

(A) L6 myoblasts were transfected with either pEGFP or GFP-tagged NGFI-B. Cells overexpressing GFP-NGFI-B showed a significant loss of mitochondrial membrane potential indicated by the reduction in red fluorescence associated with the MMP dye, tetramethylrhodamine methyl ester (TMRM).

(B) Quantification of the loss of TMRM. The * indicates significantly different from empty vector ($p < 0.0001$).

4.2.2 Overexpression of NGFI-B did not cause loss of MMP in HeLa cells

To assess whether this NGFI-B-dependent loss of MMP was unique to muscle cells, HeLa cells were transfected with either pEGFP or GFP-NGFI-B, and processed in the same way as previously described using TMRM staining. Visualization using fluorescent microscopy followed by similar quantification revealed no significant difference in TMRM fluorescence between transfected and non-transfected as well as pEGFP and GFP-NGFI-B transfected cells, indicating that the loss of MMP resulting from over-expression of NGFI-B is unique to L6 skeletal myoblasts (Figure 4.2A). Again, quantification of TMRM fluorescence revealed no significant difference between pEGFP and GFP-NGFI-B expressing cells (Figure 4.2B)


A
48hr pEGFP
48hr GFP-NGFIB
TMRM
GFP/TMRM

B

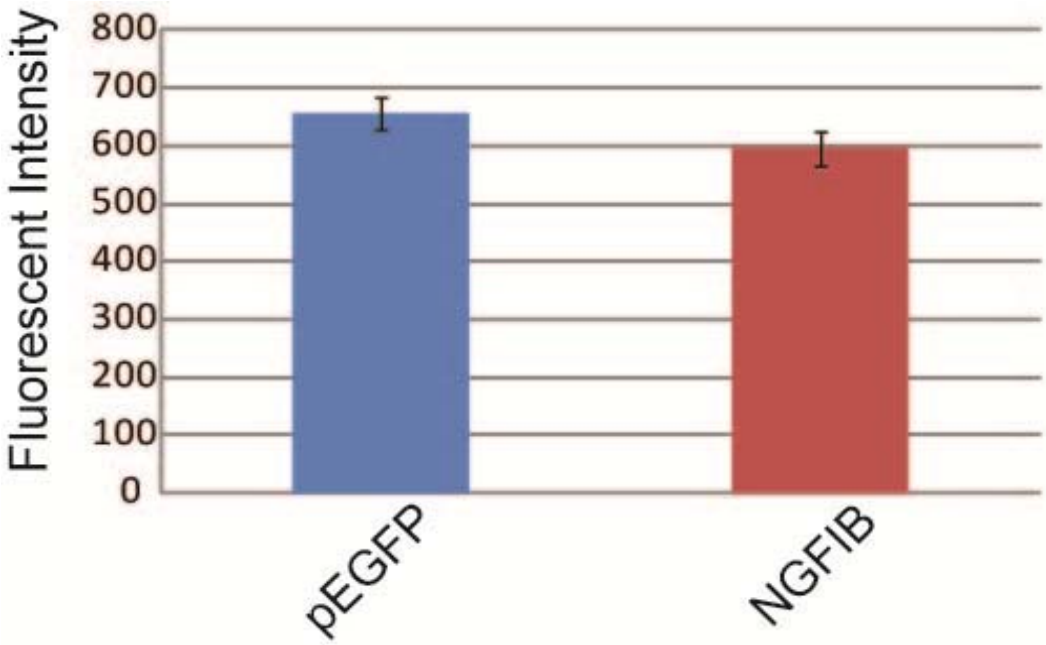


Figure 4.2 Effect of NGFI-B on MMP in HeLa Cells

(A) HeLa cells 48 hours post transfection with either pEGFP or GFP tagged NGFIB demonstrated no significant difference in the presence of mitochondrial membrane potential.

(B) Quantification of TMRM fluorescence

4.2.3 NGFI-B-dependent MMP loss is caspase-independent

To begin to understand the mechanism surrounding the observed NGFI-B-dependent MMP loss in L6 myoblasts we first established, despite the healthy morphology associated with these cells lacking a MMP, whether apoptosis was occurring. To do so, we initially treated L6 muscle cells with 50 μM of the pan-caspase inhibitor Z-VAD-FMK (BD Pharminogen) just after transfection with pEGFP or GFP-NGFI-B. The cells were treated with the same concentration of Z-VAD-FMK every day 24 hours up to 48 hours to prevent the activation of caspases and the induction of apoptosis. Cells were then stained with TMRM to determine MMP using fluorescent microscopy. Treatment with Z-VAD-FMK failed to prevent NGFI-B-dependent MMP loss (Figure 4.3).

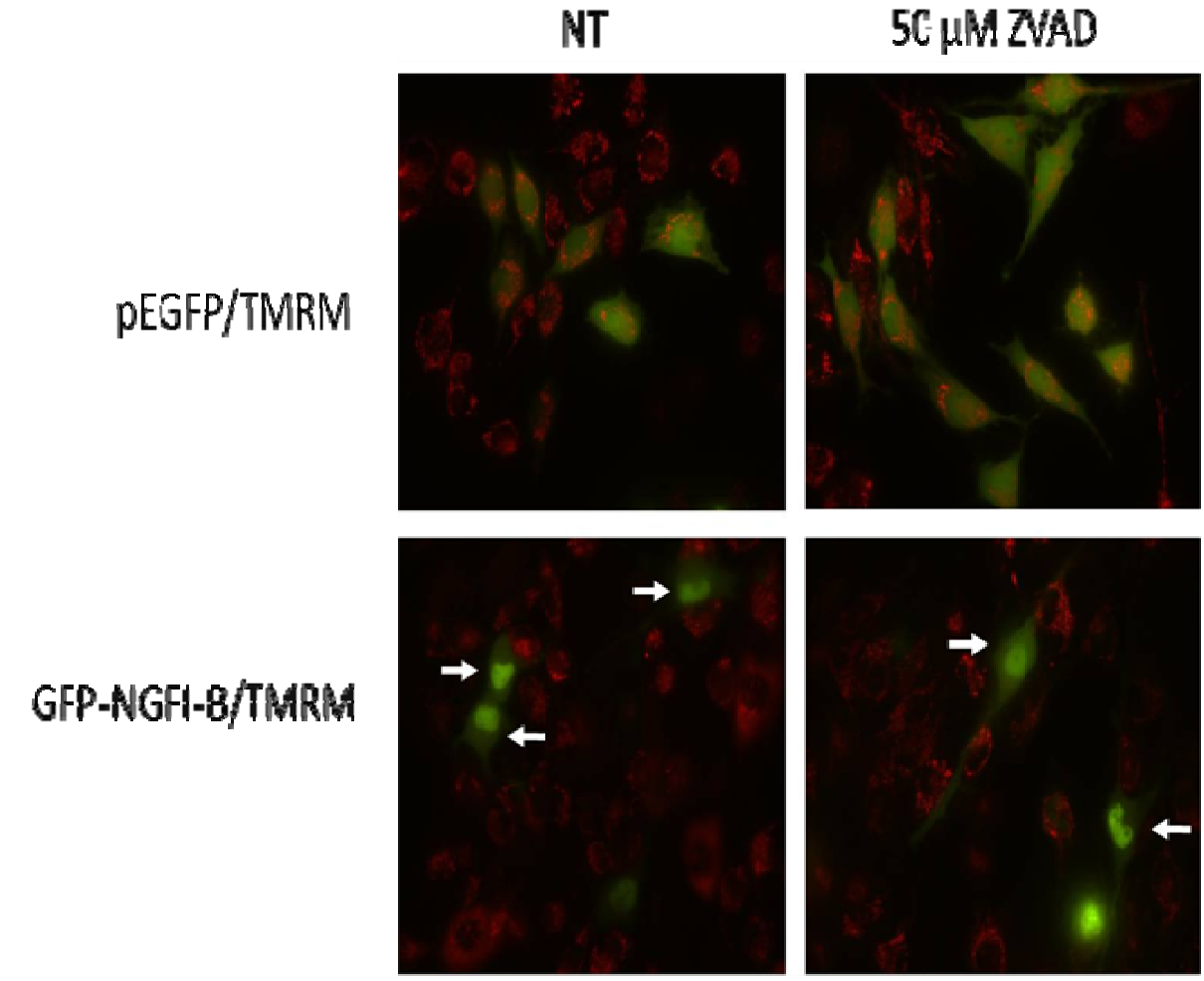
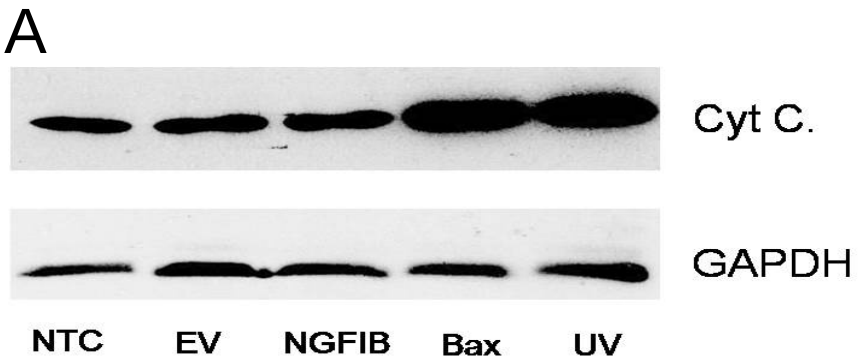


Figure 4.3 Influence of caspase inhibition on NGFI-B-dependent MMP regulation

(A) L6 muscle cells transfected with GFP-NGFI-B were treated with the vehicle control dimethyl sulfoxide (DMSO) or exposed to the pan-caspase inhibitor, ZVAD-FMK (50 µM). At 48 hours MMP was assessed in NGFI-B positive cells using TMRM and fluorescent microscopy.

4.2.4 Overexpression of NGFI-B does not cause excessive cytochrome c release

Aside from caspase activation, cytochrome c release from the mitochondria is another well-established indicator of apoptosis induction. Therefore, in order to determine if cytochrome c release is associated with the NGFI-B-dependent loss of MMP in L6 muscle cells, myoblasts were transfected with empty vector, NGFI-B-myc, or Bax-myc (positive control) for 48 hours. Another set of L6 myoblasts were also exposed to DNA-damaging UVB to provide another positive control for cytochrome c release. In each case some cytochrome c was released, however the amount released by both empty vector and NGFI-B-myc transfected cells was similar to that observed in non-transfected cells (Figure 4.4A). Larger cytochrome c bands were observed in the cytosolic fractions from both positive controls, Bax transfected and UVB irradiated (Figure 4.4A). Densitometry of the bands using the GAPDH loading control further confirmed that cytochrome c released from the mitochondria in L6 cells expressing NGFI-B were minimal compared to the two well established inducers of apoptosis and cytochrome c release, Bax and UVB treatment (Figure 4.4B).



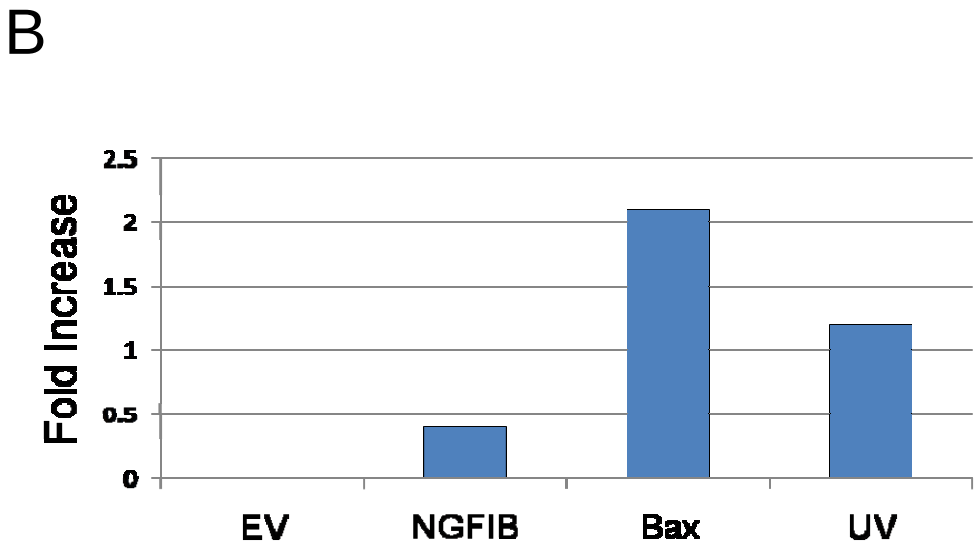


Figure 4.4 Effect of NGFI-B over-expression on cytochrome C release

(A) Cytochrome c release was determined using digitonin to collect the cytosolic fractions of L6 muscle cells transfected for 48 hours with either empty vector (EV) or NGFI-B. The pro-apoptotic factor Bax and exposure to ultra violet light (UV) for 10 minutes were used as positive controls for cytochrome c release.

(B) Densitometry was performed and all bands were normalized to the cytosolic loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and expressed as fold increase over non-transfected cells.

4.2.5 Loss of MMP is independent of the transcriptional function of NGFI-B

As a result of the even distribution of GFP-NGFI-B observed throughout the cell we next attempted to determine whether the transcriptional function of NGFI-B was important for the loss of MMP observed in NGFI-B expressing L6 muscle cells. To do so, we utilized a construct containing a truncation of the NGFI-B activation domain (NGFI-BΔAF-1) (Figure 4.5A) that was therefore unable to drive NR4A family-dependent transcription was utilized (Figure 4.5B). L6 muscle cells were co-transfected for 48 hours with pEGFP and either empty pcDNA6/myc-his vector, myc-tagged NGFI-B, or myc-tagged NGFI-BΔAF-1, in a 1:4 ratio. Cells were then stained with TMRM and GFP positive cells were assessed for the presence of red fluorescence indicating MMP. Transfection with the myc-tagged NGFI-BΔAF-1 still lowered MMP (Figure 4.5C) suggesting the mechanism by which NGFI-B lowers MMP is independent of NGFI-B transcriptional function. In fact, it appeared that the cells expressing the truncation mutant, NGFI-BΔAF-1, caused an even more robust loss of MMP at 48 hours than those transfected with full-length NGFI-B (Figure 4.5C). This is consistent with observations from other labs indicating the ligand-binding domain (LBD) of NGFI-B is all that is sufficient for NGFI-B-dependent induction of apoptosis (Lin, Kolluri et al. 2004; Kolluri, Zhu et al. 2008). Further investigation using a GFP-tagged form of the truncation mutant, NGFI-BΔAF-1, is needed to assess whether it localizes more rapidly to the mitochondria.

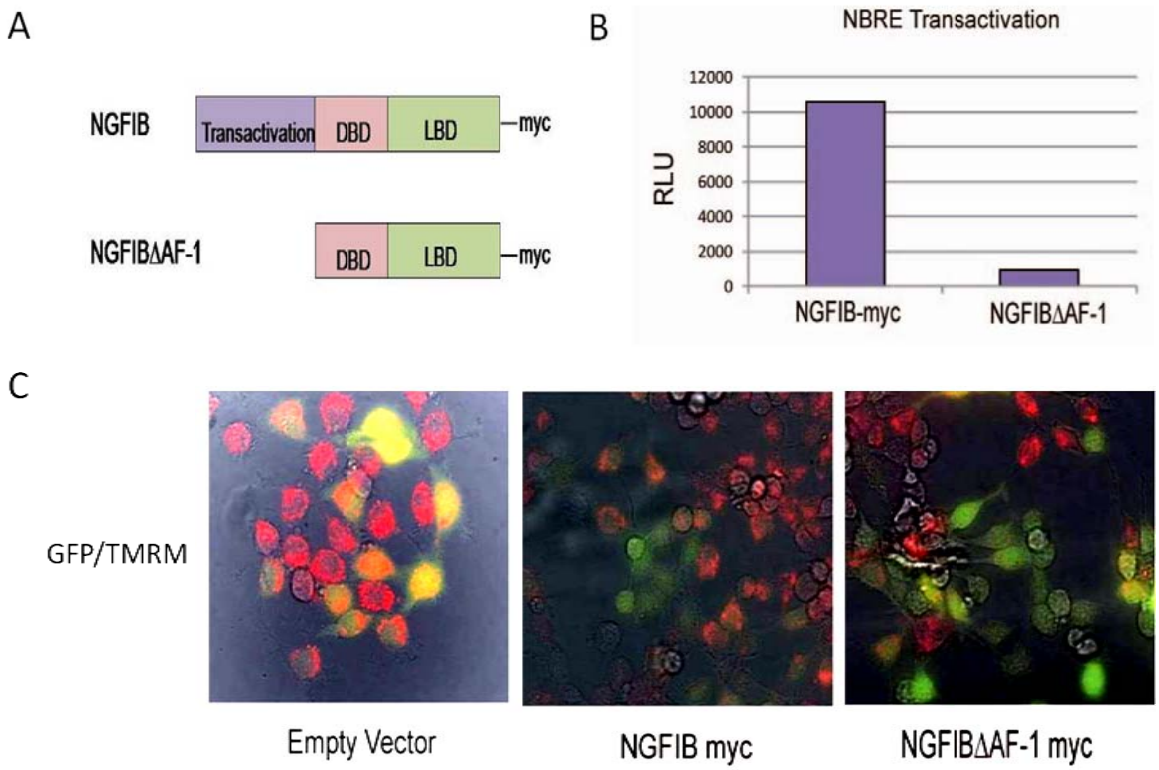


Figure 4.5 Relevance of NGFI-B-dependent gene expression on loss of MMP

(A) The activation domain (AF-1) of NGFI-B was truncated.

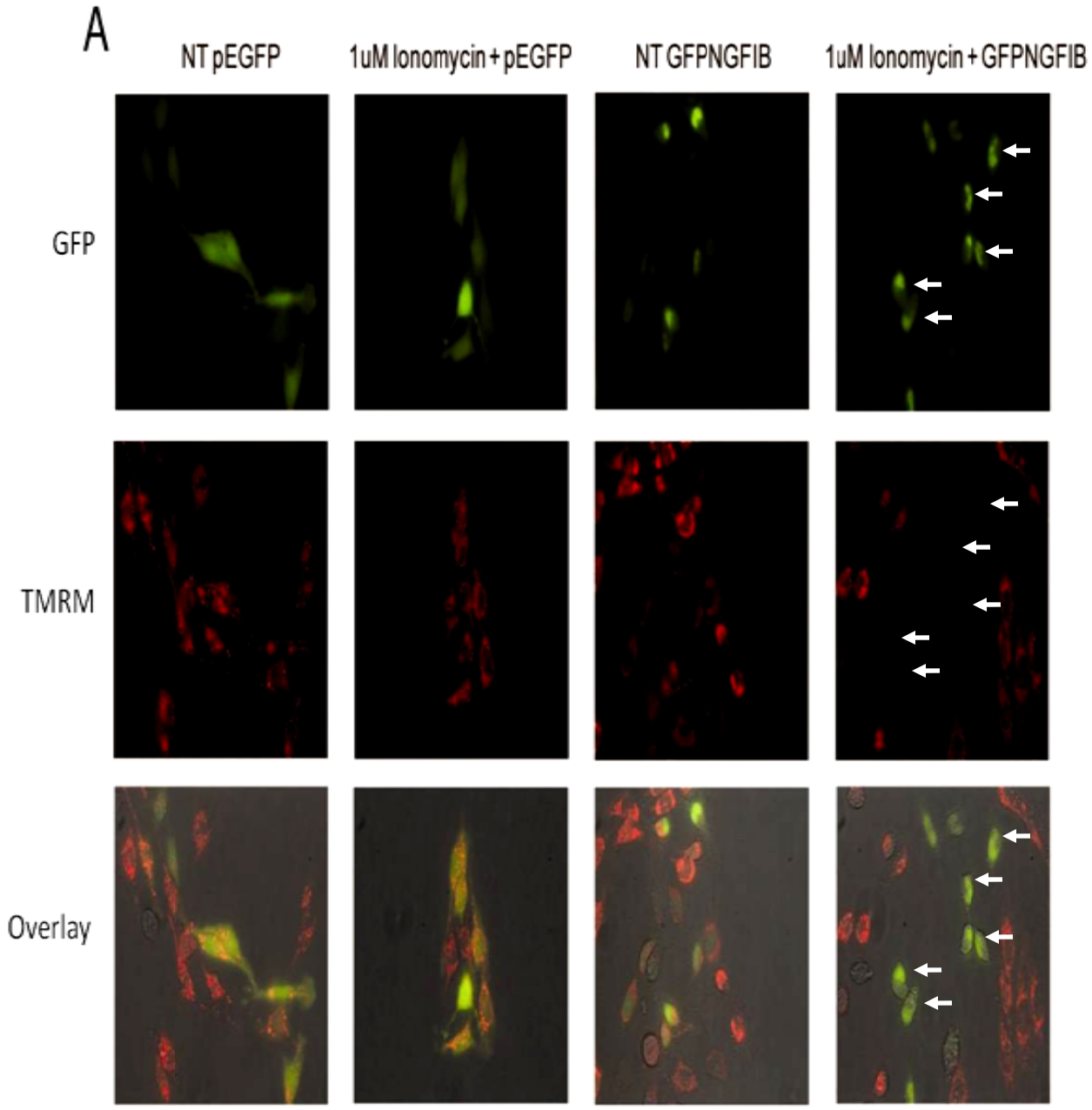
(B) Co-expression of the NGFIBΔAF-1 with the NBRE luciferase vector resulted in the loss of transcriptional activity. Luciferase expression is normalized to renilla.

(C) TMRM fluorescence in L6 myoblasts transfected with either empty vector, full length NGFI-B, or NGFIBΔAF-1. pEGFP positive cells were examined for the presence of membrane potential in the form of red fluorescence.

4.2.6 Ionomycin induces NGFI-B-dependent loss of MMP

To further elucidate the mechanism by which NGFI-B expression causes the loss or lowering of MMP in L6 myoblasts similar experiments were performed with or

without the lipid-soluble calcium ionophore, ionomycin. At concentrations of 1-10 μM, ionomycin has been shown to cause the release of calcium from several intracellular calcium stores, including the endoplasmic reticulum (ER) and the mitochondria (Brini, Bano et al. 2000). Ionomycin along with phorbol 12-myristate 13-acetate (PMA) activates protein kinase C (PKC) and simulates T-cell receptor (TCR) mediated apoptosis (Weiss and Littman 1994). Likewise, the induction of NGFI-B is also required for T-cell receptor (TCR) activation induced apoptosis (Cheng, Chan et al. 1997). Interestingly researchers observed that treatment with cyclosporine A (CspA), an inhibitor of the MPTP component cyclophilin D, not only caused a reduction in the amount of NGFI-B protein induced by ionomycin but correspondingly prevented immature T-cells from undergoing apoptosis (Woronicz, Lina et al. 1995). The involvement of the MPTP and a calcium ionophore in the regulation of TCR-mediated apoptosis provides evidence that mitochondrial calcium release could be involved in the regulation of NGFI-B-dependent loss of MMP in L6 myoblasts as well as in the induction of NGFI-B-dependent apoptosis in other cell lines. Correspondingly, we observed that treatment with 1 μM ionomycin 24 hours after transfection with GFP-NGFI-B caused a rapid, 3 hour, loss in MMP (Figure 4.6A). This same loss of MMP was not seen in cells transfected with pEGFP (EV) with or without treatment with ionomycin (Figure 4.6A). Also, significant MMP loss was not observed at 24 hours in GFP-NGFI-B expressing L6 muscle cells (Figure 4.6A) providing preliminary evidence that calcium could be involved in the mechanism by which NGFI-B regulates the MMP. Again, quantification of red fluorescence confirmed visual observations (Figure 4.6B)


A
NT pEGFP
1uM Ionomycin + pEGFP
NT GFPNGFIB
1uM Ionomycin + GFPNGFIB
GFP
TMRM
Overlay

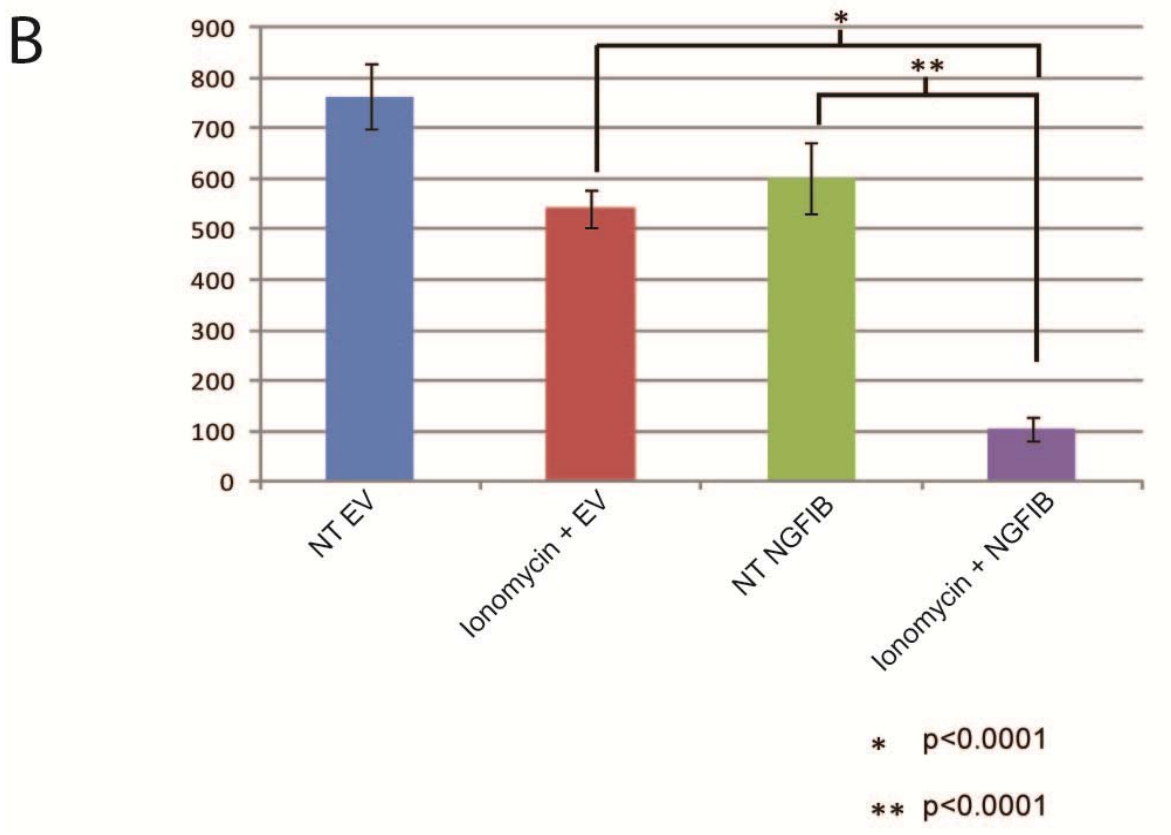


Figure 4.6 Role of calcium in NGFI-B-dependent MMP regulation

(A) Twenty one hours post transfection with either pEGFP or GFP-NGFI-B L6 muscle cells were treated with the vehicle control DMSO or with 1 μM ionomycin for 3 hours. Cells were then stained with TMRM and their membrane potentials were assessed using fluorescent microscopy. Ionomycin caused a significant loss of MMP in NGFI-B expressing cells compared to both the untreated NGFI-B and the ionomycin treated cells transfected with empty vector.

(B) Quantification of the presence of TMRM. The * indicates significantly different from empty vector ($p < 0.0001$). The ** indicates significantly different from untreated NGFI-B ($p < 0.0001$).

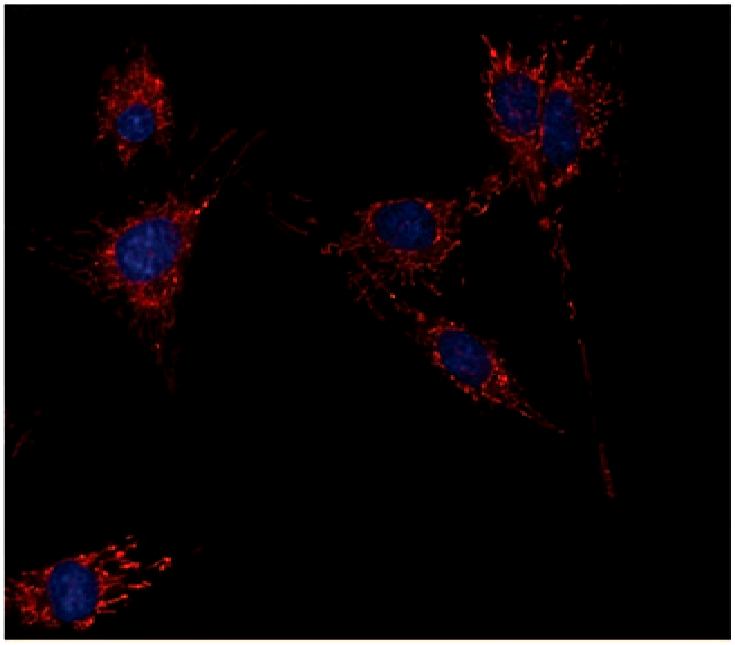
4.2.7 Stable expression of NGFI-B resulted in loss of MMP

Next, L6 stable cell lines were created using the lentiviral system described in chapter 2 and either the FG9 empty vector (EV) or the FG9 construct expressing NGFI-B to further examine the mechanism responsible for this NGFI-B induced loss of MMP. Once single clones were selected using the antibiotic, hygromycin, and screened for the presence of either the EV or NGFI-B expression, cultures were continued under normal growth conditions to prevent experimental interference by the presence of hygromycin. Stable cell lines stained with both TMRM to observe MMP and Hoechst 33342 nuclear dye to allow for clear visualization of cell presence. Remarkably, stable expression of NGFI-B in L6 myoblasts appeared to lower or cause the complete loss of MMP in all cells compared to those infected with EV (Figure 4.7A). Consistent with this data, a similar experiment using flow cytometry also revealed a dramatic reduction in the presence of red fluorescence in NGFI-B stable L6 muscle cells compared to the EV stable L6 cell line (Figure 4.7B). Interestingly, no sign of cell death or sickness appeared in the NGFI-B expressing stable cells which overall looked remarkably similar, morphologically, to the EV stable cell line.

A

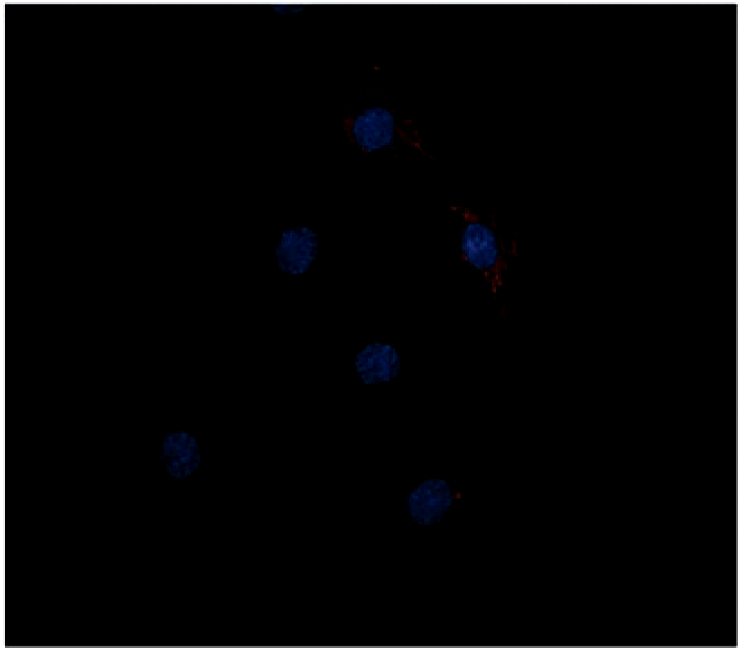
Empty Vector L6

Hoecsht/TMRM



NGFI-B L6

Hoecsht/TMRM



B

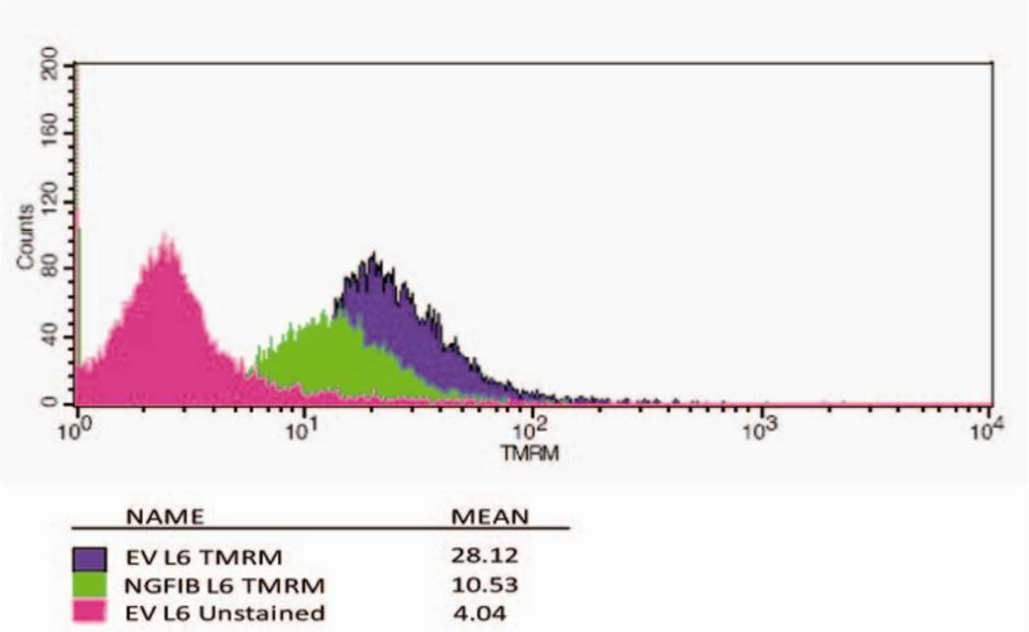


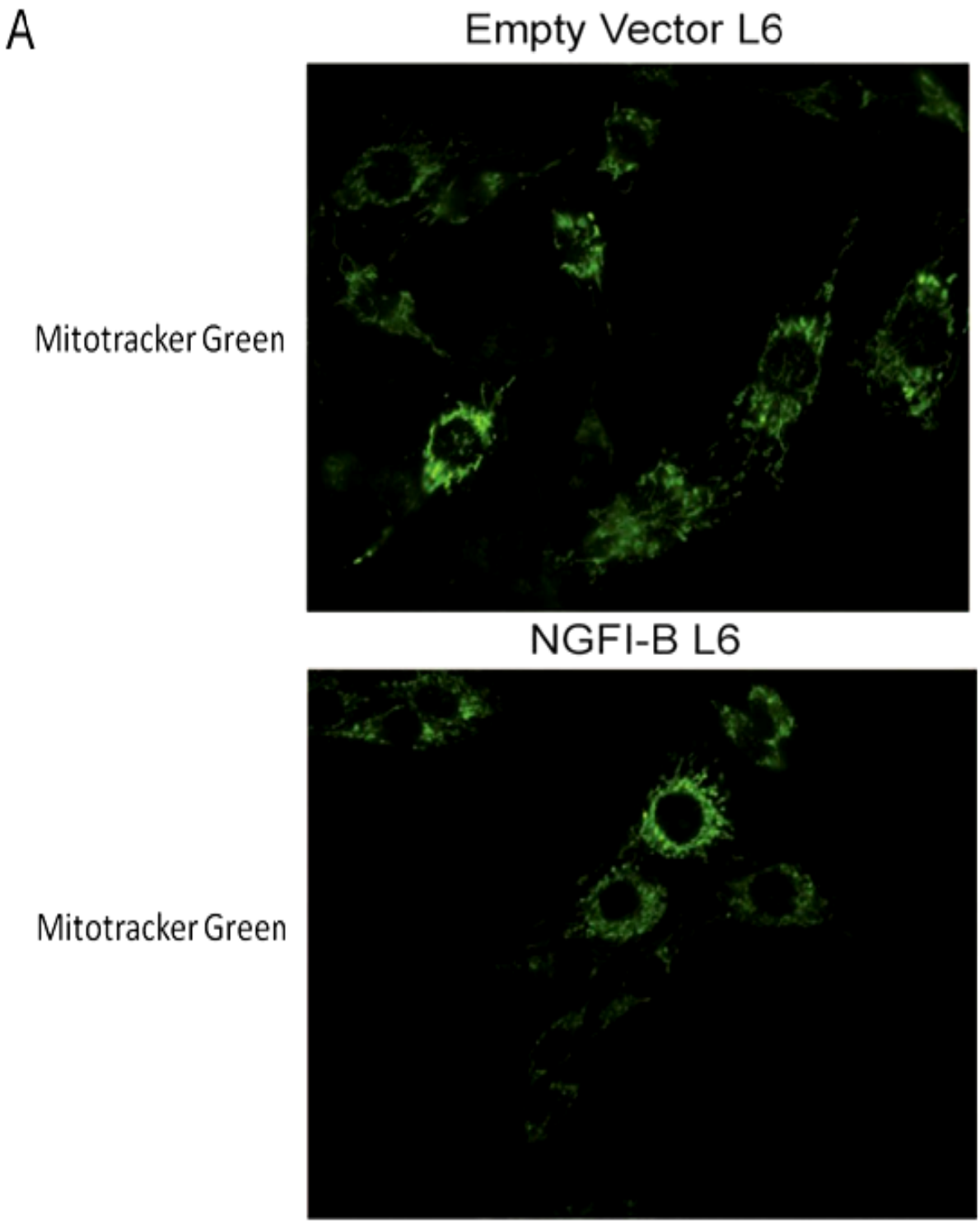
Figure 4.7 Effects of stable overexpression of NGFI-B on MMP

(A) Using the lentiviral expression system, L6 muscle cells were infected with either NGFI-B or empty FG9 vector (EV) followed by selection in hygromycin. Once colonies were screened for the stable expression of NGFIB, MMP was determined using TMRM and fluorescent imaging. The Hoechst 33342 nuclear live cell stain (4 μM, Invitrogen) was utilized to clearly visualize NGFI-B expressing L6 cells lacking MMP as well as indicating that while lacking MMP the cells remain viable.

(B) Flow cytometry was used to confirm the MMP effect witnessed during fluorescent imaging. A definitive shift to the left of NGFI-B expressing L6 cells was observed compared to that of L6 cells infected with empty vector indicating stable expression of NGFI-B does indeed lower MMP.

4.2.8 Stable overexpression of NGFI-B does not alter mitochondrial number

Further characterization of the NGFI-B stable cell line used the membrane potential-independent mitochondrial dye, MitoTracker green (25 nM, Invitrogen). Both fluorescent imaging and flow cytometry revealed no significant difference in mitochondrial mass (Figure 4.8A and B) as measured by MitoTracker green fluorescence. This suggests loss of MMP is not attributable to an overall decrease in mitochondrial number within L6 muscle cells stably expressing NGFI-B.


A
Empty Vector L6
Mitotracker Green
NGFI-B L6
Mitotracker Green

B

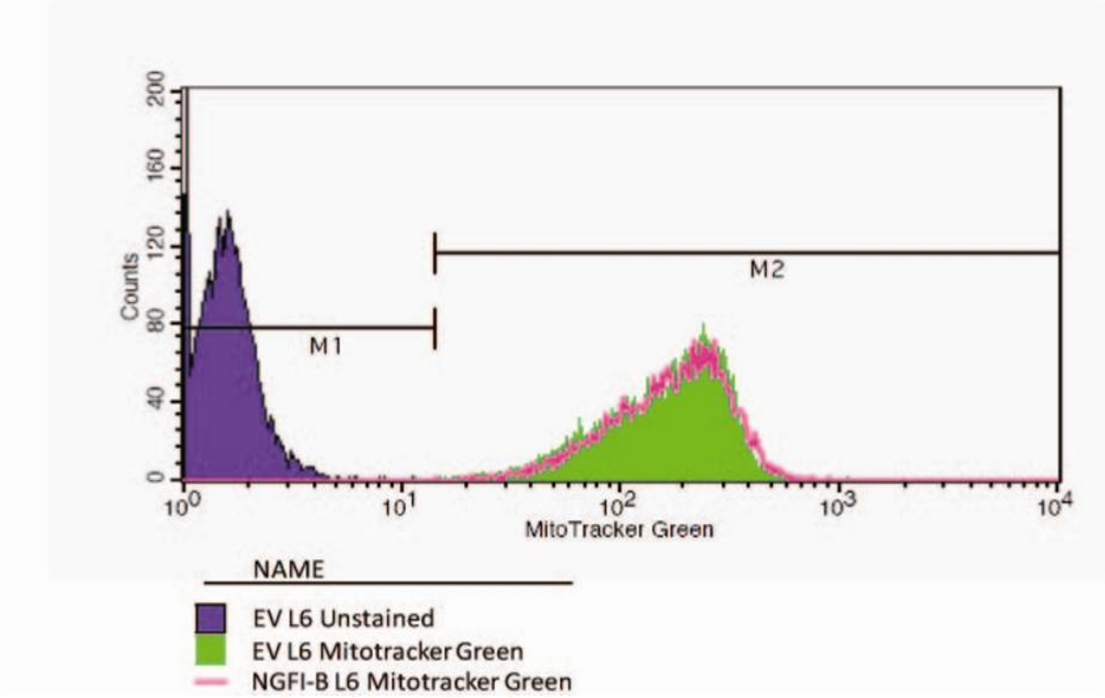


Figure 4.8 Effects of stable overexpression of NGFI-B on mitochondrial number

(A) Mitochondrial number was examined in both stable EV and NGFI-B expressing L6 cells using the membrane potential-independent mitochondrial dye, MitoTracker green. No obvious difference in mitochondrial number was visualized using fluorescent microscopy.

(B) Using flow cytometry the similarity in mitochondrial number between EV L6 cells and NGFI-B L6 cells was confirmed. Loss of MMP in NGFI-B-expressing L6 cells is not due to a change in overall mitochondrial number.

4.2.9 Stable overexpression of NGFI-B results in increased State 3 respiration

The stable cell lines were next tested for differences in oxygen consumption using a Clark-type electrode. NGFI-B L6 stable myoblasts respired more robustly than EV L6 muscle cells indicating an NGFI-B-dependent increase in normal State 3 respiration (Figure 4.9). Oligomycin was then used to inhibit the ATP synthase in order to determine whether this difference in State 3 respiration was the result of an increase in proton leak. No significant difference in State 4 respiration was observed, ruling out the involvement of proton leak through uncoupling proteins, the adenine nucleotide translocase (ANT), or flipping of protons across the lipid bi-layer. Dinitrophenol (DNP) was used at the end of every sample to determine maximal respiration and check for overall cell fitness differences between the two stable cell lines. Again no difference was observed upon the addition of DNP suggesting both cell lines were equally viable and susceptible to chemical uncoupling.

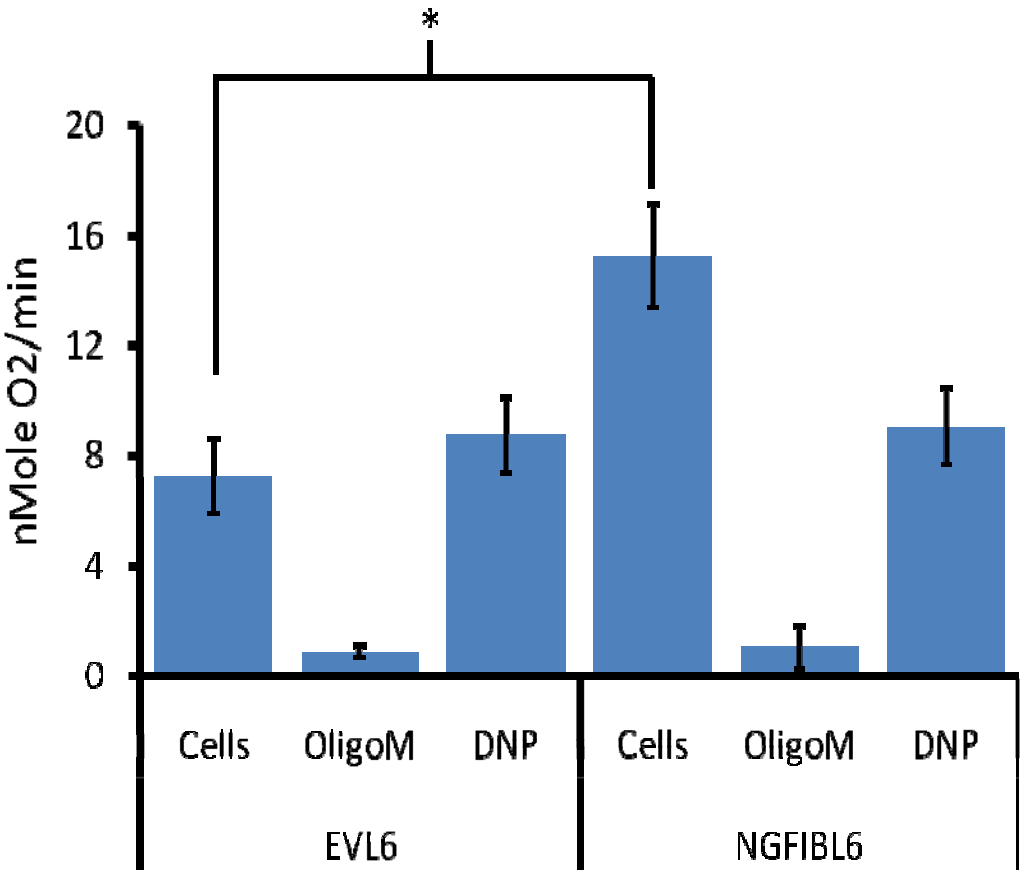


Figure 4.9 Effects of stable overexpression of NGFI-B on mitochondrial respiration

Oximetry experiments revealed L6 muscle cells stably expressing NGFI-B consumed more oxygen than EV L6 muscle cells during State 3 respiration. No significant difference between EV L6 myoblasts and NGFI-B L6 myoblasts was observed during State 4 uncoupled respiration generated by the addition of the ATP synthase inhibitor, oligomycin (1 mg/mL). 1 μL of 250 mM dinitrophenol (DNP) was added as a positive control for viability and maximal respiration at the end of every measurement. Results of oximetry activities are shown as means ± SEM (n = 3, * = $p<0.05$).

4.3 DISCUSSION

As muscle cells differentiate and fuse to form myotubes they not only become more resistant to cell death but exhibit changes in mitochondrial membrane potential (Wang and Walsh 1996; Kamradt, Chen et al. 2002). Using this knowledge and forced expression of NGFI-B in L6 skeletal muscle myoblasts we have begun to uncover the mechanism by which the nuclear hormone receptor NGFI-B regulates the MMP. We demonstrate that both transient and stable expression of NGFI-B in L6 myoblasts causes a remarkable reduction or loss of MMP without inducing cell death. We did not observe any obvious localization of NGFI-B to the mitochondria, but rather diffuse expression throughout the cell. However, experiments using a transcription-deficient mutant NGFI-B still displayed lowered MMP after 48 hours. We also reveal that stable overexpression of NGFI-B in L6 myoblasts causes a significant increase in State 3 but not State 4 respiration, thus eliminating the possibility that the loss of MMP is a result of increased proton leak through proteins such as the ANT and UCP family. Preliminary experiments utilizing the calcium ionophore, ionomycin do, however, reveal a possible role for calcium regulation in the mechanism by which NGFI-B lowers the MMP in skeletal muscle cells. Further systematic experiments using inhibitors of the MPTP such as cyclosporine A and knock-down of Bcl-2 will hopefully illuminate how NGFI-B regulates membrane potential and possibly give further insight into how NGFI-B induces cell death.

The mitochondrial targeting of the transcription factor NGFI-B through an interaction with the anti-apoptotic protein Bcl-2 has been shown to induce cell death in several cell lines (Li, Kolluri et al. 2000; Holmes, Soprano et al. 2003; Kolluri, Bruey-Sedano et al. 2003; Holmes, Soprano et al. 2004; Lin, Kolluri et al. 2004; Lee, Ma et al. 2005; Maddika, Mendoza et al. 2006). Thus far, the only information about the

mechanism by which this interaction between NGFI-B and Bcl-2 propagates cell death is that it involves a conformational change in Bcl-2 which reveals its BH3-domain (Lin, Kolluri et al. 2004). It is proposed that this converts Bcl-2 to a pro-apoptotic protein. Researchers assumed that this conversion does not cause apoptosis by resulting in direct action of Bcl-2 at the mitochondrial outer membrane but by rather through the decreased ability of Bcl-2 to inhibit the pro-apoptotic Bid and Bax. Aside from the eventual release of cytochrome c following NGFI-B mitochondrial targeting through Bcl-2, the exact mechanism by which apoptosis is induced remains vague. Interestingly, treatment of human neuroblastoma SK-N-SH cells and human esophageal squamous carcinoma EC109 and EC9706 cells with the retinoid-related molecule 6-(3-(1-adamantyl)-4hydroxyphenyl)-2-naphthalene carboxylic acid (AHPN) caused NGFI-B translocation to the endoplasmic reticulum (ER) where it interacted with ER-localized Bcl-2, initiated the release of calcium, and lead to ER stress and eventual induction of apoptosis (Liang, Bin 2007 Exp Cell Research). The ER plays a critical role in the regulation of virtually all processes in healthy cells through maintenance of intracellular calcium and the folding and processing of newly synthesized membrane and secreted proteins (Berridge, Lipp et al. 2000). Not surprising then, are the many studies which have demonstrated that disruption in the ER calcium pool is involved in the induction of ER stress and unfolded protein response (UPR) often leading to apoptosis (Bian, Hughes et al. 1997; Ferrari, Pinton et al. 2002). Similar to the mystery surrounding how the NGFI-B:Bcl-2 complex leads to cytochrome c release it, likewise, remains unclear how the interaction between NGFI-B and Bcl-2 causes calcium release from the ER and subsequent ER stress. Some studies suggest ER-targeted Bcl-2 inhibits apoptosis by preventing the efflux of calcium across the ER membrane (Scorrano, Oakes et al. 2003; Bassik, Scorrano et al. 2004; Palmer, Jin et al. 2004; Pinton and Rizzuto 2006). However, others suggest

overexpression of Bcl-2 actually causes an upregulation of ER calcium pumps and an increase in luminal calcium concentrations (Kuo, Kim et al. 1998). While further investigation of whether the same conformational change in Bcl-2 localized at the ER occurs upon interaction with NGFI-B and how this effects calcium release is needed, evidence presented thus far indicate a strong correlation between the interaction between NGFI-B and Bcl-2, calcium release, and the induction of apoptosis. Understanding of how the NGFI-B: Bcl-2 complex regulates ER stress will no doubt provide insight into how that same interaction regulates NGFI-B-dependent mitochondrial induced apoptosis.

Mitochondria are also associated with intracellular calcium storage and regulation. Maintenance of the MMP at 180-200 mV in respiring mitochondria drives the influx of calcium ions (Ca^{2+}) into the mitochondria (Rizzuto, Bernardi et al. 2000). This creates a sink for calcium and alters the availability of intracellular calcium for signaling purposes (Herrington, Park et al. 1996; Hoth, Fanger et al. 1997; Billups and Forsythe 2002). Opposite of ER stress which is a result of an efflux of too much calcium from the ER, an influx of too much calcium into the mitochondria is believed to cause the full opening of the MPTP, irreversible loss of the MMP, and the induction of apoptosis (Bossy-Wetzel, Newmeyer et al. 1998). We observed that treatment with 1 μM ionomycin 24 hours after transfection with GFP-NGFI-B caused a rapid, 3 hour, loss of MMP. This same loss of MMP was not observed in non-treated GFP-NGFI-B transfected cells at 24 hours or in pEGFP transfected cells treated or left untreated. At concentrations of 1-10 μM, ionomycin has been shown to cause the release of calcium from several intracellular calcium stores, including the endoplasmic reticulum (ER) and the mitochondria (Brini, Bano et al. 2000). Ionomycin along with phorbol 12-myristate 13-acetate (PMA) also activates protein kinase C (PKC) and simulates T-cell receptor mediated apoptosis (Weiss and Littman 1994). Likewise, the induction of NGFI-B is

also required for T-cell receptor (TCR) activation induced apoptosis (Cheng, Chan et al. 1997). Interestingly researchers observed that treatment with cyclosporine A (CspA), an inhibitor of the MPTP protein, cyclophilin D not only caused a reduction in the amount of NGFI-B protein induced by ionomycin but correspondingly prevented immature T-cells from undergoing apoptosis (Woronicz, Lina et al. 1995). The involvement of the MPTP, a calcium ionophore, and NGFI-B in the regulation of TCR-mediated apoptosis provides evidence that mitochondrial calcium release could be involved in the regulation of NGFI-B-dependent loss of MMP in L6 myoblasts as well as in the induction of NGFI-B-dependent apoptosis in other cell lines.

Skeletal muscle is a highly metabolic tissue accounting for 50% of an organism's energy expenditure and is considered the primary tissue for insulin-stimulated glucose uptake/disposal as well as paramount to the regulation of metabolism by managing circulating and stored lipids (Maxwell, Cleasby et al. 2005; Chao, Zhang et al. 2007). Consequently, muscle is now believed to be heavily involved in insulin sensitivity and other metabolic disorders. Skeletal muscle mitochondria, being crucial to the regulation of fatty acid oxidation/storage and the generation of energy have now been directly linked to obesity and the onset of Type II diabetes (Simoneau, Veerkamp et al. 1999; Kelley, He et al. 2002). Our research argues that NGFI-B overexpression can lead to the lowering or loss of MMP in skeletal muscle cells independent of cell death. While this phenomenon may be attributed to established muscle cell resistance to cell death it still sheds light on the mechanism by which NGFI-B regulates both mitochondrial bioenergetics and apoptosis. Previously, we have also demonstrated that NGFI-B induction and transcriptional activity is redox-regulated. Lowering of the mitochondrial membrane potential has been shown to increase the flux of electrons through the ETC, therefore reducing the amount of electrons slipping to molecular oxygen and the

generation of superoxide (Korshunov, Skulachev et al. 1997; Liu 1999). Therefore, NGFI-B-dependent MMP loss also provides a potentially unique method of regulating mitochondrial ROS production that could in turn regulate downstream redox targets such as NGFI-B, itself. A greater understanding of the correlation between mitochondrial bioenergetics and cell signaling pathways is critical to developing better treatments for the prevention of carcinogenesis, metabolic pathologies such as diabetes mellitus, as well as a variety of inflammatory disorders.

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